ACTA PHYSIOLOGICA SCANDINAVICA
VOL. 61. SUPPLEMENTUM 227

ANALYSIS OF IONIC TRACER MOVEMENTS DURING SINGLE HEART CYCLES

BY

ULF SJÖSTRAND

UPPSALA 1964

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AKADEMISK AVHANDLING

SOM MED VEDERBÖRLIGT TILLSTÅND AV KANSLERN FÖR
RIKETS UNIVERSITET FÖR VINNANDE AV MEDICINE DOKTORSGRAD
FRAMLÄGGES TILL OFFENTLIG GRANSKNING Å
FYSIOLOGISKA INSTITUTIONENS I UPPSALA FÖRELÄSNINGSSAL
TISDAGEN DEN 19 MAJ 1964 KL. 9 F.M.

AV

ULF SJÖSTRAND
MED. KAND., SMÅL.

UPPSALA 1964
ALMQVIST & WIKSELLS BOKTRYCKERI AB

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FROM THE INSTITUTE OF PHYSIOLOGY, UNIVERSITY OF UPPSALA, SWEDEN

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It is the purpose of the present work to describe methods for and to present some results of the relation between ionic fluxes and electromechanical activity of heart muscle (sinus venosus and atrium of the toad).

The *introduction* consists of a review of the background to these studies together with a general discussion of the problem involved.

In the *methodological section* the preparation of the tissue sample used, i.e. sinus venosus and, in some cases, atrium from toads, is described. This is followed by a description of the perfusion system and the recording methods used. Under the titles of *influx experiments* and *efflux experiments* are given experimental results together with a discussion of methodological problems, while the biological discussion is treated under the heading *general discussion*.

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Guide for some abbreviations and special terms

AC component, see pp. 54–55
base line, see p. 54
CAT, see p. 53
c-a-t, see p. 53
cpm, counts per minute
cps, counts per second
cps_s, see p. 81
Δcps_r, see p. 81
Δcps_s, see p. 81
D, diffusion coefficient
DC component, see p. 54
decimal-pip method, see p. 39
direct monitoring method, see DMM
DMM, perfusion technique, see p. 45
DMM, radioactive analysis, see p. 38
ESA, equivalent specific activity, see p. 41
intercept-interpolation method, see p. 57
kcpm, 1 kcpm = 10^3 cpm
kcps, 1 kcps = 10^3 cps
M, molar (concentration)
M, molar mass
$\overline{M}_n$, average molar mass (number average)
$\overline{M}_W$, average molar mass (weight average)
mean cycle, see p. 54
mean trend, see p. 54
monitoring pulse, see p. 39
N, number of observations by DMM, see p. 55
n, number of observations (for all experiments except DMM), (see p. 22); number of recorded impulses for radioactive analyses (see pp. 37, 41)
O_R, external surface film during silicone oil perfusion, see pp. 66–67
O_{RD}, see pp. 66–67
original value method, see p. 57
R, Ringer solution (phase)
R, long Ringer perfusion period
r, short Ringer perfusion period
S, silicone oil (phase)
S, long silicone oil perfusion period
S, standard error of the estimate
s, short silicone oil perfusion period
sampling method, see SM
SM, see p. 49
t_m, length of radioactive measuring period, see p. 45
t_r, length of Ringer perfusion period, see p. 45
trapped activity, see pp. 60–61
t_{reqv}, see p. 74
t_s, length of silicone oil perfusion period, see p. 45
$\bar{v}$, linear mean velocity, see pp. 29–30
v_{method}, see p. 37
V_o, volume of O_R, see p. 75
v_{stat}, see p. 37
x, value of single observation
$\bar{x}$, arithmetic mean of observations $x_1, x_2 \ldots x_n$

INTRODUCTION

I. *The Purpose of the Present Investigation*

Bernstein (1902, 1912) made the first attempt to explain the asymmetry in the distribution of the inorganic ions between the inside and outside of living cells. As experimental methods have developed, new and more precise data for an ever increasing number of different tissues and cell types have been obtained, making it possible to extend the theoretical treatment. Particularly important has been the development of electrophysiological recording methods (Schütz, 1936; Hodgkin and Huxley, 1939; Graham and Gerard, 1946), which has made it possible to amass data for both resting and action potentials.

The structure in the cell membrane and the driving forces which work within it are the factors with which most theories attempt to explain *resting potential* in the cell membrane (for references, see below). Ling (1962) has presented, however, a hypothesis which is essentially based on the *properties of the cytoplasm* itself. Boyle and Conway (1941) have, like Bernstein, presented a hypothesis concerning the properties of the cell membrane; their postulates were based essentially on studies of striated muscle. Important studies of the electrical properties of the cell membrane were initiated by Cole and Curtis (1939), who studied the membrane impedance during a single action potential. Goldman (1943) appears to be the first who treated the rectification properties of membranes theoretically. Passive and active transport has been treated by Ussing (1949*a*, *b*; 1951). The constant field assumption presented by Hodgkin and Katz (1949) goes back to Goldman's (1943) constant field theory and is an antecedent to the now well-known Hodgkin–Huxley theory (1952*a–d*); Teorell (1951, 1953) treated this problem also in the case for a charged membrane.

According to the Hodgkin–Huxley *theory* (1952*a–d*) the ionic distribution *at rest* is maintained by an active outward transport of sodium coupled with a very low sodium permeability. This theory is

based mainly on data obtained from the squid axon, and explains resting potential as depending on the concentration differences between intracellular and extracellular ions, mainly potassium ions, which exist in the resting state.

Teorell (1960) put forward a hypothesis in which he postulated that the electro-chemical and electro-osmotic relationships led to a bulk flow, which in its turn resulted in the separation of ions within the charged pores of the membrane. According to Teorell's view, water transport between the cellular and extracellular spaces may play an important role in the genesis of the electrical events (cf. Teorell, 1962).

In order to explain the repolarization of the cell membrane, which takes place during an *action* cycle in an excitable cell, permeability changes within the cell membrane (causing a shift from K to Na dominance) have been postulated (Hodgkin and Huxley, 1952*a–d*).

It is, however, not only events in nervous and striated muscular tissue which have attracted great interest. During the last ten years much data has been obtained concerning relationships during excitation in both the muscles and conducting system of the heart. Since Woodbury, Woodbury and Hecht (1950) first recorded intracellularly membrane potentials of a single heart fibre, much data concerning the relationships of the heart muscle cell have been presented. Further information may be obtained in recent review articles (Brooks, Hoffman and Orias, 1955; Weidmann, 1956; Schütz, 1958; Carmeliet, 1961; Trautwein, 1963).

From an analysis of published electrophysiological data, Noble (1960, 1962) developed a theory analogous to that of Hodgkin–Huxley, which takes regard of the special electrophysiological conditions in heart tissue. A theoretical discussion of the Na- and K-hypothesis has been presented also by Brady and Woodbury (1960).

Generally it may be said that most workers are agreed that the findings from a particular excitable tissue are applicable to most other types of excitable tissue.

We shall now discuss the observations which support the view that there is a connection between the electrical activity and ion fluxes in excitable tissue:

Keynes (1951) and Keynes and Lewis (1951) studied ion fluxes (Na^{24}, K^{42}) in nerve tissue and found that the rate of accumulation of the tracer in an axon was higher during stimulation than at rest;

the outflow of the tracer was also more rapid when the nerve was being stimulated. Similar results were obtained by TASAKI, TEORELL and SPYROPOULOS (1961), who studied Na^{24} and K^{42} outflow from squid axon, the rate of outflow of the tracer depending on the stimulation frequency. In studies of cardiac muscle SCHREIBER (1956) found that the K^+ outflow was related to the cardiac work done; similar findings were reported by GRUPP (1963). Reference should also be made to SEKUL and HOLLAND (1959) who studied Na^{24}, K^{42}, Ca^{45} and Cl^{36} fluxes in isolated rabbit atria and obtained an increased influx of Na^{24}, Ca^{45} and Cl^{36} and an increased K^{42} and Cl^{36} efflux with stimulation (no effect of stimulation on Na^{24} and Ca^{45} effluxes); HARRIS (1958) observed that stimulation also increased Cl^- output from frog muscle. The potassium permeability in Purkinje fibres was studied by CARMELIET (1961). HAAS, GLITSCH and TRAUTWEIN (1963) found that the rate of efflux of Na^{24} was greater from a beating than from a non-beating heart.

It is further known that acetylcholine and vagus stimulation influence ion transport in the heart. BURGEN and TERROUX (1953) showed that acetylcholine and similar substances acted on the heart by increasing the permeability for potassium ions. This was confirmed, among others, by SEKUL and HOLLAND (1959), HUTTER (1961) and DANIELSON (1962, 1964). FATT and KATZ (1951) interpreted changes in the motor end plate potential in frog muscle by assuming that acetylcholine produced a non-selective increase of ionic permeability. KLEIN and HOLLAND (1958) claimed that acetylcholine increases the transmembrane flux of Na^+ and K^+ (studies of Na^{24} and K^{42} on isolated rabbit atria). SEKUL and HOLLAND (1959) obtained no effect of acetylcholine on the fluxes of Ca^{45} and Cl^{36} in isolated rabbit atria. DANIELSON (1964) found an increased potassium transport in the toad heart as a result of vagal stimulation, but no effect on Na^+ or Br^- ions was observed. These latter observations concerning bromide are in agreement with HUTTER (1961). WADELL (1961) studied potassium movements in auricular muscle under the influence of adrenaline, which he claimed increased both influx and efflux of potassium. HAAS and TRAUTWEIN (1963) reported an increase of sodium efflux induced by adrenaline in the heart of frog.

Several workers have studied influx and/or efflux rates for sodium and potassium. HARRIS (1957) studied K^+ movements in frog muscle as did HODGKIN and HOROWICZ (1959). BURROWS and LAMB (1962)

studied Na^+ and K^+ fluxes in tissue cultures of heart muscle from chicken embryo. Potassium movements in heart muscle have also been studied by Rayner and Weatherall (1959) and Humphrey and Johnson (1960).

Data about the calculated mean flux (net value of simultaneous inward and outward fluxes) for the ions studied during the whole cycle of activity, given as the number of micromoles transported per unit of time or per activity cycle and weight of tissue can be found in many of the works cited above (for a review reference may be made, for example, to Carmeliet, 1960, p. 26 ff.). *Calculations of the quantitative fluxes* from tracer experiments may be misleading if there is some uncertainty about the question of maintenance of a uniform specific activity during the experiment.

The experiments discussed above cannot give information about the *direct* relationships between the ion flux and the different phases of a *single* action cycle. It seems clear that is particularly desirable to obtain detailed information about this *direct relationship between ion flux and the single action cycle*. Experiments with this aim have been carried out, for example on squid axon by Spyropoulos and Tasaki (1961). It appears probable that such information can also be obtained on other excitable tissues. Only some isolated attempts to determine the ion flux during a single action cycle in heart muscle have been published. The experiments of Wilde and O'Brien (1953) are well known; the studies were made on isolated turtle heart, and an increased K^{42} efflux was found during the later part of the T-wave of the electrocardiogram. Danielson, Öbrink and Sjöstrand (1961, 1962) studied Na^{24} and K^{42} efflux from an isolated auricle and/or sinus venosus of the frog; Na^{24} and K^{42} effluxes showed cyclical variations in relation to the heart cycle. Na^{24} efflux had a maximum at the end of the electrical activity, while the K^{42} efflux appeared to be increased during the whole of the electrical activity. Lorber *et al.* (1962) studied K^{42} efflux from a piece of frog ventricle and found an increased efflux during and of approximately the same duration as the action potential.

The present work attempts a more detailed and more *direct* qualitative analysis of ionic flux in an excitable tissue (heart muscle) during the single action cycle (electro-mechanical). The possibilities for such an analysis depend to a great extent on matters of a purely technical nature. The question of the selection of the experimental

preparation and the general principles of attacking the problem must first be considered.

The ideal preparation should have a long action potential which, for example, by cooling can be further prolonged. It should further have a good surface volume relationship in order to obtain good exchange conditions; it should also be of a suitable size, and sufficiently large for measurements to be made (this latter factor involves a compromise with the former). *The sinus venosus in the toad* seemed to be a rather suitable experimental object, though it has a large extracellular space and like the other parts of the heart, mechanical activity; both these properties complicate the study of membrane processes. From a general methodological point of view, the sinus venosus and/or auricle of the toad were considered to be suitable; both these cardiac regions are easily prepared and have a good viability.

The only possibility of studying *radioactive influx* in a preparation is to follow the changes in the radioactivity in the preparation itself. Studies of the influx of ions have been published (Harris, 1953; Johnson, 1957; Shanes, 1958; Carmeliet, 1961; Haas, Glitsch and Trautwein, 1962, 1963; Tasaki, 1963). The methods reported, however, cannot be used if it is desired to analyse the radioactivity of the preparation under time intervals so short that several observations can be made during a single cycle of activity (for example a single heart cycle).

In *studying the radioactive influx* the preparation must, on the one hand, have the possibility of taking up radioactive tracer, and, on the other hand, the measurement of its radioactive content must take place when it is not present in a radioactive bathing solution. The problem is such that the preparation should be in contact with a radioactive bathing solution of high specific activity (the background activity $\gg$ the activity of the preparation), and during very short time intervals it should be analysed for its radioactive content when the background activity is very low (the background activity $\lessapprox$ the radioactivity of the preparation). The principles for such a discontinuous analysis together with preliminary results have been discussed by Sjöstrand (1962, 1963); further the problem has also been discussed by Danielson and Sjöstrand (1963). If the preparation is mounted in a perfusion chamber (see Fig. 19, p. 38), which in its turn is placed in a NaI(Tl)-crystal, the gamma activity can be analysed continuously. By alternate perfusion with radioactive Ringer's

solution (high specific activity) and a low-viscosity silicone oil (hydrophobic and not radioactive) the preparation comes into contact with the isotope during the Ringer perfusion period, while during the silicone oil perfusion period it is surrounded by silicone oil in the perfusion chamber, whereby the background activity is reduced to a very low level. During the silicone perfusion period the radioactivity recorded is mainly that present in the preparation. By a continuous recording of the electrophysiological events in the preparation (in these experiments the ECG), the discontinuous radioactivity measurements can thus be analysed in terms of the electrical events.

Studies of the radioactive efflux are technically much simpler. The pioneer work was made by Wilde and O'Brien (1953). The efflux studies discussed above have all been made in technically similar ways, and are all in principle different modifications of the "wash-out" technique (Danielson, Öbrink and Sjöstrand, 1961, 1962; Lorber *et al.*, 1962). This method was also used by Spyropoulos and Tasaki (1961) on squid axon.

II. *The Sinus Venosus and the Atrium of the Heart of the Toad*

Sinus venosus or atrium from the toad (*Bufo bufo*) was used. Concerning the anatomical details reference should be made to Ecker–Wiedersheim (1904, p. 269), Skramlik (1921) and Uchiyama (1959). Fig. 1 shows a semi-schematic diagram of the toad heart.

The main object for the present studies has been the sinus venosus, but when it was desired in a simpler way to study the normal physiological relationships the atrium has also been used (the electrical and mechanical properties; studies of the equilibrial volumes and the selection of suitable test molecules for such studies). In addition use of the atrium and sinus venosus enables more certain comparisons to be made with the results reported in the literature.

a) *Microscopic anatomy*

A short review of the microscopic anatomy has been given by Kisch (1961) who maintains that the *sinus venosus* has a layer of striated muscle which has similar histological properties as the muscle in other parts of the toad heart. The endothelial cells have a large number of mitochondria (similar to the mammalian heart). Between the epithelial cells and the muscle cells are a large number of longitudinal and transverse collagen fibres. Capillaries are never found between the muscle cells. In a later work (Kisch, 1962) it was shown that the endothelial layer in certain places has a thickness of only 0.1 μ or less, and in certain places the sarcolemma of the nearest muscle fibres was separated from the endothelial covering cells by a subendothelial stratum. The protofilament in a muscle cell has a diameter of about 180 Å. The *atrium* has to a great extent a similar construction; the muscle cell layer is, however, considerably thicker. Lindner (1957) reported the thickness of the sarcolemma to be 150–250 Å for ventricular muscle of *Rana temporaria*, and claimed that the endothelial cells correspond to the vascular endothelium. The extracellular

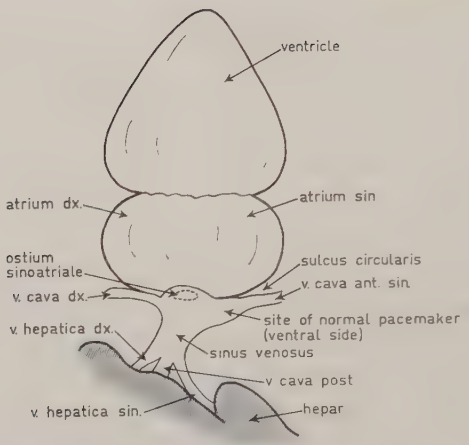

Fig. 1. Toad heart (*Bufo bufo*), semidiagrammatic. To expose the sinus venosus the auricles and ventricle have been lifted cranially.

space begins in the subendocardial region and communicates with tissue spaces between the muscle cells. The heart muscle cells have a greatest thickness (in the region of the cell nucleus) of between 2.5 and 6 μ; their diameter, however, can be as low as 0.45 μ elsewhere. In cross section the muscle cells consists to about 2/3 of fibrillae and to about 1/3 of cytoplasm. Morphologically the boundary between the intracellular and extracellular spaces is difficult to define. A value of less than 5 μ was given by Hutter and Trautwein (1956) for the heart muscle cells in the frog, and by Iwamoto (1959) for sinus venosus fibres in the toad. Haas, Glitsch and Trautwein (1963) give a diameter of about 4 μ for atrial muscle cells in the frog.

Briefly may also be mentioned the interesting observations of Simpson and Oertelis (1962) that in the sarcolemma of the sheep heart the surface membrane complex seems to be indented with each Z-band in the subsarcolemmal myofibrils. There appeared to be two different tubular systems within the cells (sarcotubular systems), of which one had relatively thick walls which were identical with the sarcolemma, while the other had thinner walls. The thick-walled tubules seemed to be invaginations of the sarcolemma, which passed right across the cells. In serial sections it was possible to show that this tubular system was in continuity with the extracellular space. The transverse tubules and their communication with the extracellular space imply not only advantages for the propagation of the im-

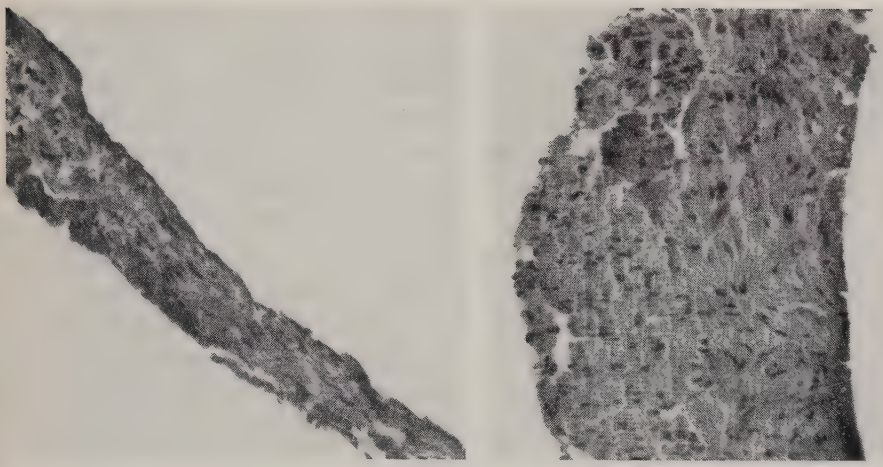

FIG. 2. Histological section of an inverted sinus venosus (left) and atrium (*Bufo bufo*). The tissue was fixed in Bouin's solution, dehydrated and embedded in paraffin; 10 μ thick section stained with hematoxylin and eosin. × 200.

pulse, but also that the total area of the sarcolemma must be considerably greater than would appear from the external area of the cell itself. This might mean that large ion fluxes could occur in a very short time. In what way the extracellular fluid perfuses the tubular system is however unknown. SPIRO (1962) has also observed invaginations in the sarcolemma.

In certain muscle tissues nearly all myofibrils are in contact with the external sarcolemma, for example in cells which have a very small diameter, i.e. toad myocardium (FAWCETT and SELBY, 1958; GRIMLEY and EDWARDS, 1960). In these cases it would appear that a tubular system of the type present in the mammalian heart was not required.

Fig. 2 shows the general morphology of an inverted sinus venosus and atrium from a toad (*Bufo bufo*).

b) *Preparation of the toad heart*

After decapitation of the toad the heart was dissected free and the sinus venosus and/or atrium was separated from the surrounding connective tissue. Both the anterior venae cavae were ligated within a few mm of the entrance into the sinus venosus. In a preparation of the latter it was separated from the atrium by cutting along the circular sulcus (Fig. 1). A plastic catheter was introduced through the sino-atrial

orifice and passed out through the posterior vena cava. Both hepatic veins were ligated. The sinus venosus could then be cut out from the toad, and was then transferred from the plastic catheter to the ECG-lead catheter as shown in Fig. 22 (p. 46) which is fixed to the part of the U-tube which served for both influx and efflux experiments (see chapter V). The sinus venosus was thereafter turned inside out (the endothelial side = the outside), and secured to the catheter at both ends. When being fixed to the ECG catheter in the perfusion chamber the sinus venosus was stretched a little so that the amplitude of the contractions was reduced considerably. The endothelial side of the sinus venosus could, with this technique, be washed without variations in tension occurring in the wall of the preparation (a rise in tension in the wall of the sinus venosus increases the spontaneous activity; cf. PATHAK, 1958; MAINWOOD, 1962). When the atrium was used the preparation was made in a similar way, i.e. it was dissected free from the bulbus arteriosus. A cut was then made along the atrio-ventricular groove, after which the catheter could be pushed in through the right atrium and out through the sinus venosus. The preparation was then cut along the circular sulcus as before.[1] The atrium was transferred in a similar manner to the ECG catheter in the perfusion chamber, turned outside in (left atrium became situated in the inverted right atrium) and secured to ECG catheter. In this case also the preparation was stretched in order to reduce movements during contraction.

The sinus venosus or atrium prepared in this way beat spontaneously. In normal oxygenated frog Ringer's solution (solution I, see below), spontaneous beating continued for as long as several days. In all experiments the tissue was allowed to rest for about 30 minutes after the preparative manipulations, in order to allow the potassium flux from the damaged cells to cease (BAMMER and ROTSCHUH, 1952*a*, *b*; ROTSCHUH, 1952) and also that the "healing over" could have time to take place (DÉLÈZE and WEIDMANN, 1963).

The size of the tissue preparation is given as a rule as follows in terms of the wet weight. The length mounted on the ECG catheter was as a rule 9–10 mm. The diameter varied from 1.5 mm (sinus venosus) to 2.5 mm (certain atrial preparations).

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[1] An atrial preparation will only beat spontaneously with an intact circular muscle bundle at the sino-atrial junction; this is part of the sino-atrial conduction system of the amphibian heart (UCHIYAMA, AKUZAWA and IWAMOTO, 1961).

c) *Normal physiology*

Nutrition

The toad heart has no coronary system and the heart muscle normally obtains its nutriment by a direct transport of soluble substances from the plasma in the heart lumen through the sponge-like structure of the wall. Perfusion of the endothelial side of the sinus venosus or the atrium simulates therefore the normal way of nutrition for the cells of the preparation. The geometric conditions, in particular for the sinus venosus, seem to fulfil the demand as stated by HILL (1928) for oxygen supply to an actively working muscle preparation.[1] PAGE and SOLOMON (1960) give for cat heart papillary muscle the value of $r_0' = 0.6$ mm (+37°C), (see footnote). In the avascular sinus venosus the relations must therefore be even better since the total wall thickness is as a rule of the order of 100 μ (see Fig. 2; cf. also HUTTER, 1961). KEYNES (1954) points out that with K studies the preparation should not have too large dimensions.

The site of the pace-maker

Already in 1897 ENGELMANN studied where the heart contraction began. IWAMOTO (1959) reviewed the studies on the localization of the primary pace-maker in the sinus venosus and reported studies which suggested that it was localized to the ventral part of the opening of the left anterior vena cava into the sinus venosus (see Fig. 1). HUTTER and TRAUTWEIN (1956) claim that in the amphibian heart (toad) the primary pace-maker is normally situated in the left side of the ventral part of the sinus venosus.

The resting and action potentials, ECG[2] and mechanical activity

The resting and action potentials of the preparation at the perfusion temperature used with influx and efflux experiments were

[1] The equation derived by HILL gives the maximal radius of r_0' for a cylindrical muscle, which receives an adequate oxygen supply through the diffusion through the medium as

$$r_0' = \sqrt{4ky_0/a},$$

where k is a constant for the diffusion of oxygen through the muscle ($cm^2\ min^{-1}$, atm.); $k = 1.4 \times 10^{-5}$ at 20°C and increases about 1 per cent per degree C (KROGH, 1919), y_0 = the concentration of oxygen in the medium (the atmosphere), a = the rate of consumption of oxygen in ml/cm^3 tissue, per min.

[2] ECG as used here means the electrical activity recorded with a surface electrode (surface electrogram).

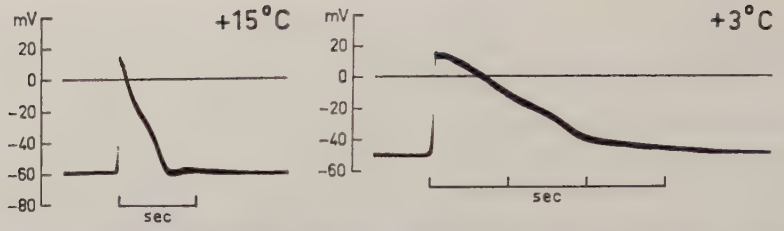

Fig. 3*a*. Membrane potential (intracellular micro-electrode) from a cell in an inverted, spontaneously beating sinus venosus (non-pace-maker region) of *Rana pipens*. Temperatures +15°C and +3°C.

studied. Figs. 3*a* and 3*b* show an intracellular recording of the action potential in an inverted, spontaneously beating sinus venosus from *Rana pipens* (two different cells in the same preparation). The recordings in Figs. 3*a* and 3*b* are in essential agreement with those reported in the literature (Hutter and Trautwein, 1956; Iwamoto, 1959; Uchiyama, Akuzawa and Iwamoto, 1961). For recording the action potential in the tissue preparation used for influx and efflux studies, a simple type of suction electrode was used (see chapter IV, A, c, 2); Fig. 4 shows such a recording of a spontaneously beating sinus venosus. The temperature dependence of the diastolic depolarization can be clearly seen in the figure (cf. Trautwein, Gottstein and Federschmidt, 1953; Couraboeuf and Weidmann, 1954). In order to obtain a longer action potential the perfusion was cooled in the ion flux experiments which will be described. Cooling[1] leads to a lower resting potential, a longer plateau in the action potential, which however is not essentially altered in other details (Woodbury, Hecht and Christopherson, 1951; Trautwein, Gottstein and Federschmidt, 1953; Heintzen, 1954; Hecht, 1956; Weidmann, 1956; Heintzen, Kraft and Wiegman, 1956).

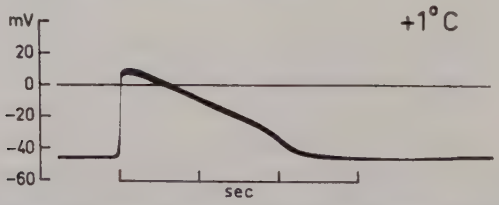

Fig. 3*b*. Membrane potential (intracellular micro-electrode) of another cell in the same preparation as Fig. 3*a*. Temperature +1°C.

[1] The efflux of K^{42} from non-beating atria is less at a lower temperature of the soaking solution (Haas and Glitsch, 1962).

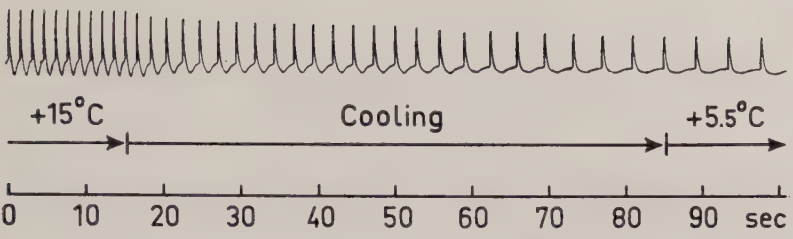

Fig. 4. Action potential (suction electrode) of an isolated, inverted, spontaneously beating sinus venosus (pace-maker or near pace-maker region) during cooling from +15°C to +5.5°C.

During an influx or efflux experiment only the ECG was recorded but from that the length of the action potential could be deduced. This can be seen in Fig. 5, which shows an ECG and action potential (suction electrode technique) in a spontaneously beating sinus venosus, and in Figs. 6 and 8 on a spontaneously beating atrium. The relationship between action potential and ECG has been treated by Hecht (1956) and Sano, Tsuchihashi and Shimamoto (1960), among others.

The mechanical activity of the preparation was also of interest. Fig. 6 shows the ECG, action potential (suction electrode) and the mechanogram (isometric) of a spontaneously beating atrium. Fig. 7 shows similar recordings from a spontaneously beating sinus venosus. The mechanical activity of the ventricular muscle of *Rana temporaria* has been described by Heintzen (1954) and Heintzen, Kraft and Wiegman (1956).

Table 1 (p. 22) summarizes the data regarding the duration of the action potential (measured by the suction electrode), the time between

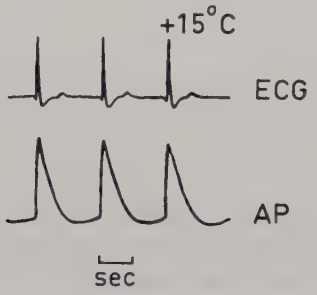

Fig. 5. Simultaneous recording of ECG and action potential (suction electrode) from an isolated, inverted, spontaneously beating sinus venosus.

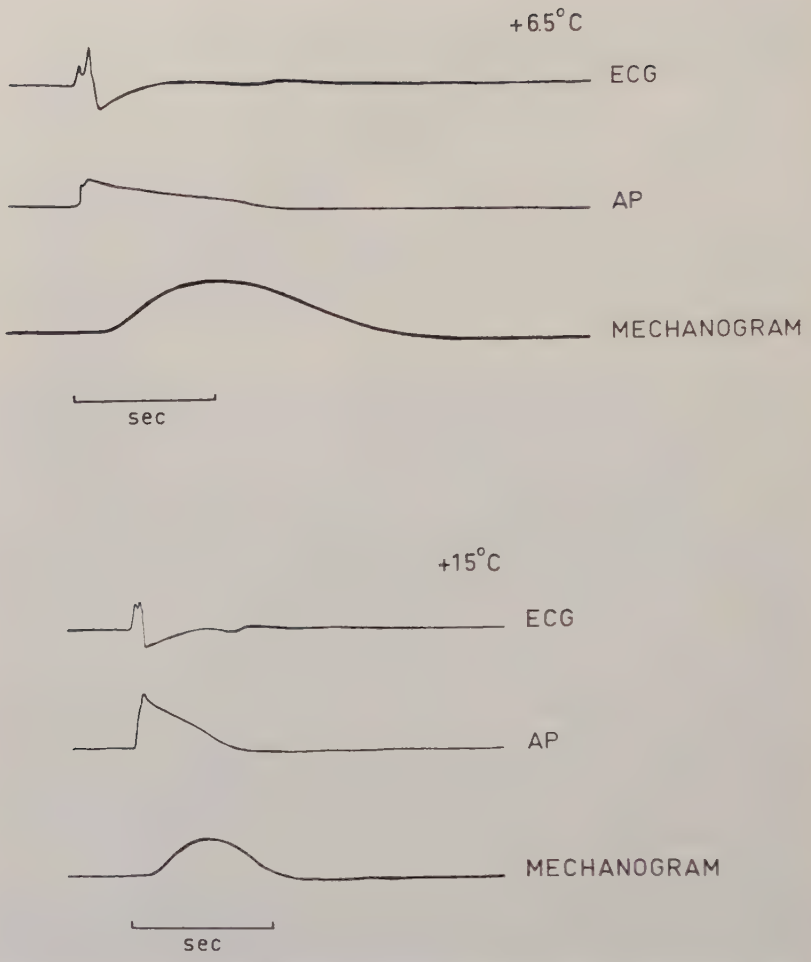

FIG. 6. The ECG, action potential (suction electrode) and mechanogram (isometric) of an inverted, spontaneously beating atrium at +6.5°C and +15°C.

depolarization (assessed from the ECG) and the beginning of the mechanical activity and also the time between depolarization and the maximum contraction of the inverted atria. In the case of the inverted sinus venosus of the toad the time interval between depolarization (ECG) and the beginning and the maximal values of the mechanical activity are given. The durations of the action potentials in the atrium are somewhat shorter than those given in the works cited above, where the investigations were performed on the ventricle musculature.

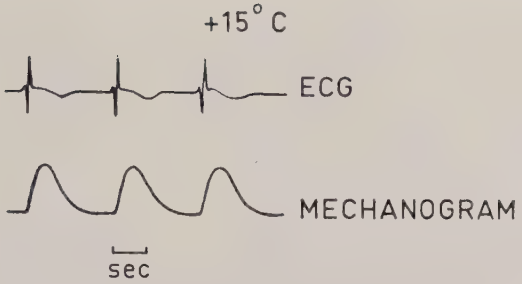

Fig. 7. The ECG and mechanogram (isometric) in an inverted, spontaneously beating sinus venosus.

Preliminary investigations on the ventricle muscle with the methods used here gave, however, somewhat higher values for the duration of the action potential. It seems therefore that the action potential in the preparation used is in fact somewhat shorter than in ventricular muscle. Shortening of the duration of the action potential with oxygen deficiency is known (Trautwein, Gottstein and Dudel, 1954), an explanation which can be rejected in this case.

Has the isolated heart preparation a synchronous electrical activity?

Since the aim was to study ion fluxes in relation to electrical and mechanical activity, it was of great importance that the electrical activity of the preparation should be synchronous in all its parts. Since as mentioned above the sinus venosus has a pace-maker localized at the site of the entrance of the left anterior vena cava, it seems probable that impulse propagation (propagation of depolarization) occurred through the preparation. Two questions were thus of interest:

1. The conduction velocity of the preparation (the conduction velocity of linear propagation through the preparation) and
2. The relationship between the ECG and the action potential in different parts of a spontaneously beating preparation (propagation of depolarization from a pace-maker whose location is not known in any particular case).

The conduction velocity of the preparation was determined by a method which is described in chapter IV, D, b, and the values for three isolated atria are given in Table 2. Heintzen (1954) reported at +5.5°C in ventricle muscle in the frog a conduction velocity of 5 cm per sec. Heintzen's value agrees with that given by Benthe

TABLE 1. *Relations between ECG and mechanogram (atrium and sinus venosus); duration of action potential (atrium).*

Temp. °C	Atrium: Exper. No.	$t_{ic}-t_{id}$ sec	n	Range	$t_{mc}-t_{id}$ sec	n	Range	Duration AP sec	n	Range	Sinus venosus: Exper. No.	$t_{ic}-t_{id}$ sec	n	Range	$t_{mc}-t_{id}$ sec	n	Range
+ 6.5	630418	0.29	27	0.24–0.33	1.17	27	1.02–1.52	1.41	10	1.34–1.46	630510 III	0.10	15	0.05–0.16	0.83	15	0.77–0.90
+ 6.5	630420 I	0.24	28	0.17–0.32	1.02	28	0.94–1.06	1.67	8	1.62–1.80	630514 I	0.09	44	0.05–0.16	0.84	44	0.76–1.00
+ 6.5	630425 I	0.16	16	0.12–0.20	1.01	16	0.93–1.13	1.71	10	1.66–1.76	630514 III	0.13	14	0.03–0.20	1.04	14	0.93–1.15
+ 6.5	630425 II	0.17	7	0.14–0.19	0.97	7	0.94–1.01	1.71	7	1.66–1.74	630514 IV	0.11	19	0.07–0.14	1.07	19	1.00–1.14
+ 6.5	630426	0.14	4	0.12–0.16	1.07	4	1.06–1.10	1.44	4	1.40–1.46	—	—	—	—	—	—	—
	Mean:	0.20		Mean:	1.05		Mean:	1.59			Mean:	0.11		Mean:	0.96		
+ 8.0	630426	0.12	5	0.11–0.14	0.83	5	0.82–0.84	1.18	5	1.16–1.20	—	—	—	—	—	—	—
+15.0	630418	0.20	28	0.17–0.25	0.63	28	0.57–0.68	0.78	19	0.74–0.82	630414 II	0.10	23	0.07–0.12	0.54	23	0.50–0.60
+15.0	630420	0.17	36	0.14–0.20	0.57	36	0.52–0.62	0.80	28	0.76–0.84	630514 III	0.14	15	0.09–0.20	0.61	15	0.57–0.68
+15.0	630425 I	0.16	8	0.14–0.18	0.75	8	0.71–0.80	0.80	8	0.76–0.82	630514 IV	0.12	19	0.08–0.15	0.58	19	0.54–0.63
+15.0	630425 II	0.12	4	0.11–0.13	0.44	4	0.43–0.44	0.83	4	0.82–0.84	—	—	—	—	—	—	—
+15.0	630426	0.10	7	0.09–0.12	0.61	7	0.58–0.63	0.90	7	0.86–0.94	—	—	—	—	—	—	—
	Mean:	0.15		Mean:	0.60		Mean:	0.82			Mean:	0.12		Mean:	0.58		

A summary of the results from isolated, spontaneously beating preparations of atrium and sinus venosus (see chapter IV, A, a, b, c). The perfusion temperature is given for each series of measurements (each experiment represents measurements on one preparation); the number of measurements in each series is denoted by n. The length of the action potential (duration AP) was deduced from suction electrode recordings. t_{id} is the time at which depolarization occurred (assessed from the ECG recording), while t_{ic} and t_{mc} correspondingly represent the times of onset and maximal value of the mechanical activity. $t_{ic}-t_{id}$ is therefore the interval between depolarization and the beginning of the mechanical activity, and $t_{mc}-t_{id}$ the interval to the maximal contraction value.

TABLE 2. *Conduction velocity measured at +5.5°C (isolated atrium).*

Exper. No.	Minimal conduction velocity (cm/sec)	No. of determinations	Range
630327	7.5	11	6.0–10.0
630328	6.0	26	4.6–10.0
630410	6.8	37	4.3–10.3
Mean	6.8		

This table gives a summary of results from isolated spontaneously beating atria which were stimulated electrically after approximately each 5th spontaneous beat 1–2 sec before the next expected would normally occur. Propagation of depolarization recorded with suction electrodes (chapter IV, D, b). Each experiment represents measurements on one preparation.

(1956). The only value of the conduction velocity of the atrium is that of ENGELMANN (1895), i.e. 9 cm per sec at room temperature.

The relationship between ECG and action potential in different parts of an inverted, spontaneously beating atrium is shown in Fig. 8. The

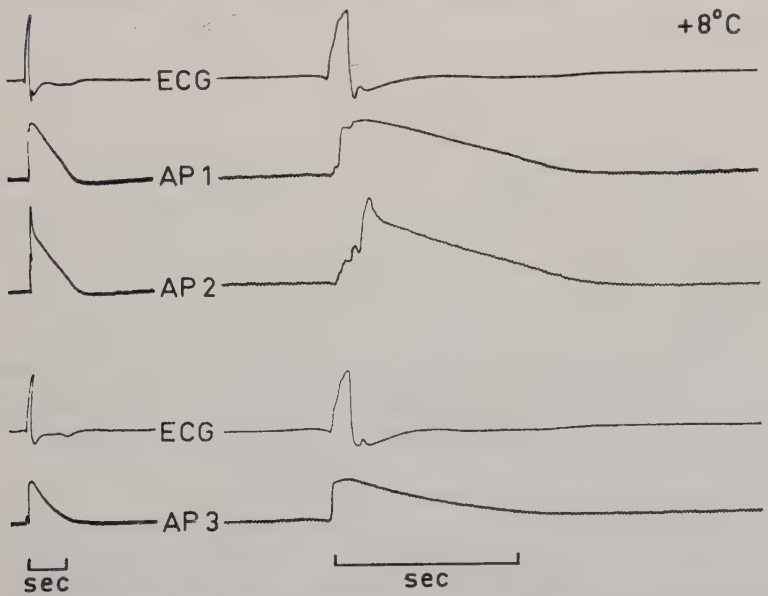

FIG. 8. The ECG and action potential (*AP*, suction electrode) of an inverted, spontaneously beating atrium at two different paper speeds (for method, see Fig. 15, p. 34). *AP* 1 and *AP* 2 were recorded at the same time as the ECG above. The lower ECG was taken later, at the same temperature, when *AP* 3 was recorded.

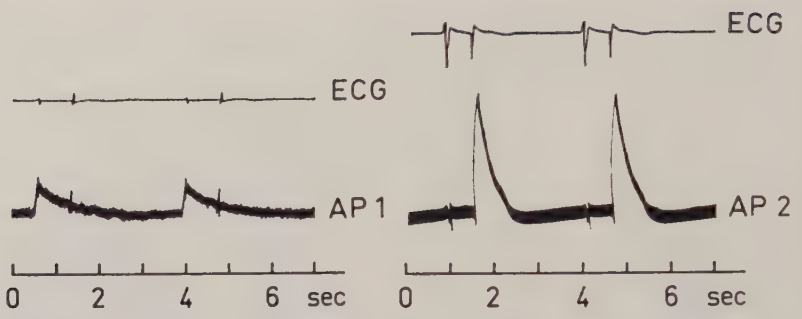

Fig. 9. The ECG and action potential (suction electrode, cf. Fig. 15, p. 34) from an inverted, spontaneously beating atrium. The left ECG and *AP* 1 were recorded during Ringer perfusion, and the records on the right during silicone oil perfusion.

figure shows simultaneous recordings of the ECG and action potentials from the upper (AP 1) and lower parts (AP 2) of the preparation; in the lower curves the ECG was also taken from a combined action potential lead (AP 3) with the method described in chapter IV, D, b, Fig. 15.[1] It can be seen that depolarization occurred practically simultaneously in the whole preparation. That the method used in the experiment of Fig. 8 was sufficiently sensitive to resolve two different electrical activities in the same preparation is evident from Fig. 9, which shows suction electrode recordings from the upper (AP 1) and the lower (AP 2) parts of another preparation with simultaneous ECG recording. This preparation had two electrical activities, one resulting from the primary pace-maker and which clearly triggered the low parts of the preparation. AP 1 was recorded during Ringer perfusion, and AP 2 during silicone oil perfusion.[2]

There seem to be good grounds for accepting that the isolated spontaneously beating atrium or sinus venosus (preliminary studies showed that similar relationships applied to sinus venosus) exhibits very nearly synchronous electrical activity over the whole preparation.

[1] The experiment of Fig. 8 gave a conduction velocity of 7.5 cm per sec at +5.5°C, when the preparation was stimulated in the upper part, according to the method which is described in chapter IV, D, b. When the preparation was allowed to beat spontaneously, the result shown in the figure was obtained.

[2] With regard to Fig. 9 attention should also be drawn to the differences in ECG registration obtained with the Ringer perfusion (preparation surrounded by a volume conductor) and that obtained with silicone oil perfusion (preparation surrounded by an insulator).

Further it is apparent (see Fig. 9) from the ECG recording whether the preparation has one or more electrical activities.

Summary: The macroscopic and microscopic anatomy of the sinus venosus and atrium in the frog and toad are reviewed. The electrical activity appears to be synchronous in all parts of the preparation even at a low temperature (about +5°C). The mechanical activity also appears to be well defined. From the ECG of the preparation the length of the electrical activity can be deduced.

III. *The Perfusion System*

As was briefly mentioned in chapter I, in the influx experiments the preparation was alternately perfused with a radioactive Ringer solution and a low viscosity silicone oil. If the latter has a linear flow rate sufficiently great to give a strongly turbulent flow, it would be expected to wash away the greater part of the Ringer solution which remained around the preparation.

In both influx and efflux studies the preparation was mounted, as described in chapter II, b, on an ECG catheter (diameter 1.2 mm) which was fixed in a polyethylene U-tube as shown in Fig. 22 (p. 46). With an influx experiment the perfusion system was arranged in such a way that the preparation was situated in a long U-loop of polyethylene tubing, which constituted a perfusion chamber (Fig. 10).[1] In an efflux experiment the preparation was mounted as shown on the right side of the figure (Fig. 22), and was perfused in the opposite direction to that with an influx study.

In both influx and efflux studies the perfusion system shown schematically in Fig. 11 was used; *Ri*, *Ra* and *S* are storage vessels for inactive Ringer solution, active Ringer solution and silicone oil, respectively. From each storage vessel (polyethylene) a polyethylene tube passes through a counter-current cooler to the two-way tap which can be seen in the lower part of the figure. In both influx and efflux experiments perfusion was performed with a high (constant) linear mean velocity. In addition in the influx studies an alternating perfusion with Ringer solution and silicone oil could be made. On these grounds the type of perfusion system shown in Fig. 11 was used: The perfusant was driven through the system by air pressure which was mediated through a tube system in connection to the storage vessels *Ri*, *Ra* and *S*. The air pressure used was maintained by a reducing valve at a level of about 1550 mm Hg (absolute pressure)

[1] The inner diameter of the perfusion chamber was 3 mm in the part where the preparation was placed (sinus venosus); the tubes leading to this part of the perfusion chamber had an inner diameter of 1.7 mm.

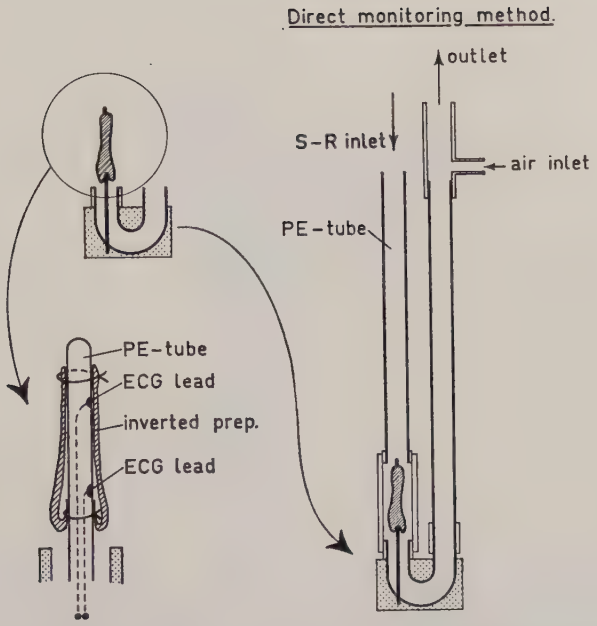

FIG. 10. The perfusion chamber used for the direct monitoring method (DMM). For further details cf. legend of Fig. 22 (p. 46).

and held constant with a 735 mm high mercury column, which in the event of an over-pressure allowed the air to leak out of the system.

The alternate Ringer-silicone oil flow periods in the influx experiments were obtained by a rapidly working two-way valve, which is shown diagrammatically in Fig. 12. The valve had inlets for both silicone oil and Ringer solution, and as shown in Fig. 11 the *inlet R* could be used for either inactive or radioactive Ringer solution. The valve (made of perspex) had an easily movable piston, which was driven by two serial coupled DC solenoids at each end with a combined effect of 0.5 kp (type TGB 4, Firma Magnet-Schultz GmbH, West Germany). The valve system could be washed through the *by-pass outlet R* and test samples of Ringer solution taken during the silicone oil perfusion period. The two-way valve was operated electronically (see Fig. 20, p. 39). The shortest perfusion period obtainable with this valve was 50 msec.

In an influx experiment the Ringer solution and silicone oil which had passed the preparation in the U-tube were collected in a collecting

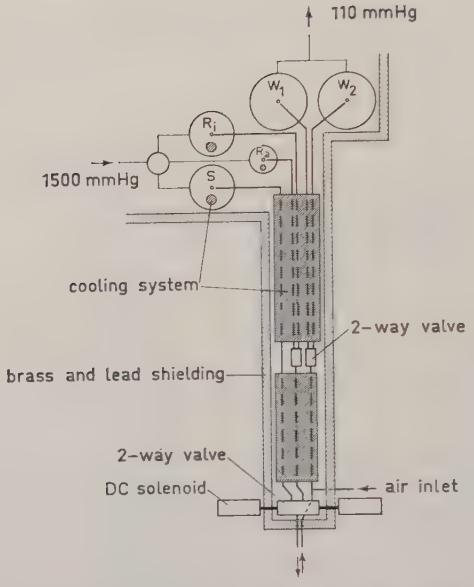

FIG. 11. Schematic drawing of the perfusion system (supply and effluent for perfusing chamber) used in both influx and efflux experiments. The pressures given are the absolute pressures in mm Hg used in influx experiments. The storage vessels are S (silicone), R_i (inactive Ringer) and R_a (active Ringer). W_1 and W_2 are the two return vessels. The latter were disconnected in an efflux experiment where the effluent from the chamber was led directly to sampling tubes in a fraction collector. Further operating details are given in the text.

vessel via a return tube. In the efflux experiments the perfusate was collected in test tubes mounted in a fraction collector. The return system used in the influx experiments is shown schematically in Fig. 11, where two waste collecting vessels (polyethylene), W_1 and W_2 are drawn. To obtain as far as possible similar perfusion and pressure conditions with both influx and efflux studies for the same perfusion pressure in the *Ri-Ra-S* system, a sucker was applied in the return system in the influx experiment. In order to minimize the radioactivity in the return tubes (to reduce the background activity during the influx experiment) they were in communication with the atmospheric pressure (air inlet in Fig. 11). By this means and with the underpressure applied to the return system the increased resistance to flow in this part of the system can be compensated.

To obtain a low background in an influx experiment it is necessary that practically all 'activity' should be some distance away from the

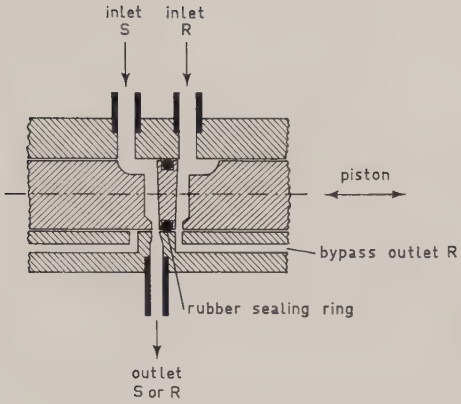

FIG. 12. Diagram of the two-way valve giving alternate Ringer-silicone oil flows. The piston movement required to switch over was 3 mm.

scintillation crystal. The supply vessels for the radioactive solution and the return vessels for both Ringer and silicone oil were therefore situated at an adequate distance from the crystal. All supply and return lines were of minimal dimensions (polyethylene tube). For health reasons the radiation shielding must also be sufficient for large amounts of activity (about 50 mC Na^{24}, with a high peak at 2.76 MeV) and also protect against the highest energy beta radiation (K^{42}, 3.6 MeV). All radioactive containing vessels and lines were first sheathed in brass (3000 mg/cm^2) which absorbs all beta energies under 4.0 MeV, and round this lead bricks (55,000 mg/cm^2). To lessen the danger of radioactive dissemination with a leakage or a possible explosion in the system, all tubes were double walled.

In order to obtain an idea of the physical relationships obtaining in the perfusion chamber during an influx study, the linear mean velocity of the Ringer solution and silicone oil during perfusion was studied at different temperatures. For an estimate of how the perfusion velocity varied depending on the size of the preparation, experiments were made both with and without the preparation mounted in the perfusion chamber. The velocities of the Ringer-silicone and silicone-Ringer phase boundaries (acceleration-deceleration process), were also studied both in the presence and absence of the preparation (see chapter IV, D, a). From Fig. 13 it can be seen that the linear mean velocity (in the incoming tube) with pure Ringer or silicone oil perfusion was about the same when the perfusion chamber was empty

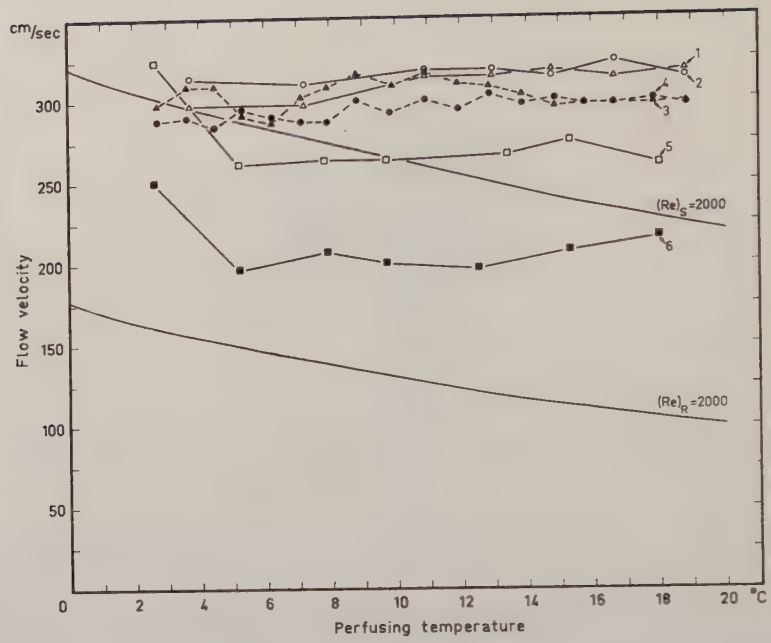

Fig. 13. The mean linear velocities ($\bar{v}$) of Ringer solution and silicone oil in the supply lines to the perfusion chamber mounted as shown in Fig. 22 (p. 46) for the direct monitoring method. The pressures were as shown in Fig. 11. The mean flows were:

without tissue preparation in the chamber
- (1) Ringer solution $\bar{v} \approx 311$ cm/sec
- (2) Silicone oil $\bar{v} \approx 316$ cm/sec

with a sinus venosus (wet weight = 20 mg) in the chamber
- (3) Ringer solution $\bar{v} \approx 303$ cm/sec
- (4) Silicone oil $\bar{v} \approx 294$ cm/sec

The velocity of the Ringer-silicone phase boundary was determined as illustrated in Fig. 21 (p. 43) without a preparation (curve 5) and with a preparation (curve 6). The critical mean velocities for appearance of turbulent flow in an "ideal" tube system containing Ringer solution (Re_R) or silicone oil (Re_S) are given there. A mean flow velocity of $\bar{v} \approx 300$ cm/sec corresponded to a volume flow of about 7 ml/sec.

as when a preparation was mounted in it. At $\bar{v} \approx 300$ cm/sec the perfusion volume was of the order of 7 ml/sec. The flow rate did not appear to vary significantly with the temperature. The pressure conditions in the perfusion system were as shown in Fig. 11 (p. 28). Fig. 13 shows that the Ringer-silicone phase boundary velocity was greater with the perfusion chamber empty. It may be concluded that viscosity changes of Ringer solution or silicone oil at different temperatures had no importance in the perfusion system.

Fig. 13 also shows the critical mean velocity for Ringer solution

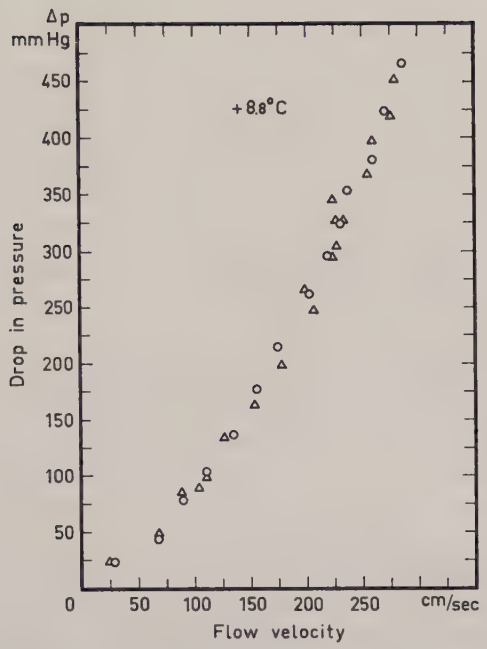

FIG. 14. The pressure difference (Δp) between the part of the perfusion chamber where the tissue was mounted and the "air inlet" in Fig. 11 (p. 28) in relation to the mean linear flow velocity ($\bar{v}$) for Ringer solution (triangles) and silicone oil (circles).

and silicone oil, at which turbulent flow appears in an ideal tube system (Reynold's number = 2000).[1] It can, however, be expected under all circumstances that there is a turbulent flow in the perfusion chamber with influx studies, since all non-ideal systems have a tendency to give turbulent flow (Reynold's number approaches unity).

Fig. 14 shows the pressure drop (Δp) between that part of the perfusion chamber in which the preparation is mounted and the air inlet in Fig. 11 (p. 28) in relation to the linear mean flow velocity ($\bar{v}$) for silicone oil or Ringer solution (the flow velocity is determined as in chapter IV, D, a, 1). According to KRONIG (1954) the flow in the perfusion chamber at a perfusion velocity of about 300 cm/sec is in

[1] In calculating these lines the data for Ringer solution (approximately = water) were obtained from the Handbook of Chemistry and Physics ed. 41, p. 2181, and for silicone oil (3 cS at + 25°C) from the Midland Silicones Ltd. Technical Data Sheet G 1 ed. 6, June 1961.

that part of the pressure difference–flow velocity diagram which corresponds to strongly turbulent flow ($\Delta p \propto \bar{v}^2$).

The temperature of the perfusion system was regulated with an accuracy of ± 0.1°C, and the maximal temperature difference between the inactive or active Ringer solution and the silicone oil during alternate perfusion was 0.2°C.

The silicone oil used was a polydimethyl-siloxane (Midland Silicones Ltd., MS 200, 2–3 cS at +25°C). Further information concerning this can be found in the Midland Silicones Ltd., London, England, "Technical Data Sheet G 1, ed. 6, June 1961", and only certain essential relevant properties will be discussed here: Compared with organic fluids the silicone oil is very stable and has a density at +25°C of about 0.87 for 2 cS ($M = 385$, cf. sucrose = 342). It has a very low surface tension, a vapour pressure of 1 mm Hg at about +55°C and is strongly hydrophobic (non-polar liquid) its solubility in water being less than 0.2 %. Because of its very marked hydrophobic properties silicone oil does not dissolve ions (see Danielson and Sjöstrand, 1963). Its solubility for gases is good, and in general the solubility is better than in water (for air the factor is about 10). The specific resistance is so high that it can be regarded as an insulator. Among the chemical properties of silicone oil it may be mentioned that it has a neutral reaction and is very resistant to oxidation. The toxicity of the oil used in these experiments is very low (Rowe, Spencer and Bass, 1948). The toxicity for red blood cells has been studied by Lindgren and Zade-Oppen (1964); no hemolysis occurred when red cells were shaken together with silicone oil. Its low toxicity is evident also by the fact that in all experiments that were carried out with Ringer-silicone oil perfusion no toxic effect on the heart preparation was observed.

IV. *Methods*

A. General recording methods

a) *The ECG*

The ECG lead was taken from the epicardial surface of the preparation (see Fig. 15, p. 34; the position of the ECG catheter in the perfusion chamber is shown in Fig. 22 p. 46). The ECG was then recorded via a Grass ECG preamplifier 5P4 (time constant 0.2 sec; Figs. 15, 20), and a Grass Model 5 Polygraph Pen Recorder.

b) *The mechanogram*

The preparation was mounted on the ECG catheter as shown in Figs. 15 (p. 34) and 22 (p. 46, sampling method). That part of the perfusion chamber which led away the perfusant in efflux experiments was not connected; the Ringer solution was able to run away over the surface of the preparation.

For measurement of the mechanogram a thin nylon thread was fastened to one end of the preparation. This thread passed to a thin metal strip on each side of which two strain gauges were fixed (the metal strip was held fast on a stand). These strain gauges functioned as the arms in a Wheatstone bridge, which was supplied with a stable direct voltage. The movements of the metal plate could thus be transduced to changes in potential which were registered with the Grass Polygraph recorder. The response of the system was linear within the region used.

c) *The action potential*

1. *Micro-electrode technique*

This part of the work was carried out in Dr. H. Antoni's laboratory in the Institute of Physiology (Head: Professor A. Fleckenstein), University of Freiburg i. Br., West Germany. The apparatus and method was as described by Antoni, Engstfeldt and Fleckenstein (1960).

2. *Suction electrode technique*

This was done essentially as according to the method described by Schütz (1931), (see Fig. 15). The suction electrodes consisted of thin polyethylene tube (inner diameter 0.4 mm) and this had a polyethylene

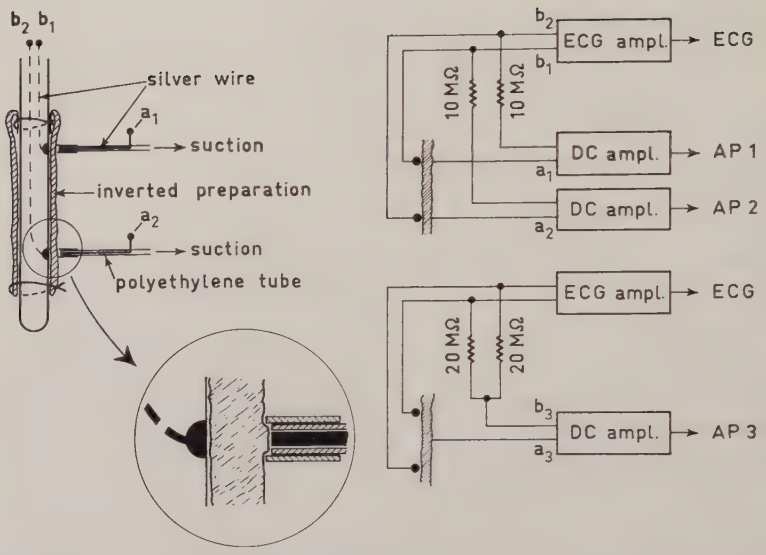

FIG. 15. The suction electrode technique. The left part of the figure shows the details of the electrode tip and the measuring sites. On the right is a semi-schematic drawing of the circuit.

collar which projected a short distance and which served to give a tighter seal with sucking. The electrode lead was a silver wire (diameter 0.1 mm) which passed inside to the end of the narrow polyethylene tube (a_1 and a_2 in Fig. 15). The Grass DC preamplifier 5P1 (time constant 0.2 sec) and Grass Model 5 Polygraph Pen Recorder were used for recording. The slow frequency characteristic of the latter, however, made a faithful reproduction of the action potential impossible. Since, however, in most cases it was only the duration of the action potential that was of interest, the recorder was satisfactory for this purpose.

d) *Weighing*

Analysis tubes and samples were weighed on an analysis balance with an accuracy of 0.1 mg.

B. Chemical analyses

a) *Sugar determinations with the anthrone method*

Dreywood's anthrone method (DREYWOOD, 1946) was used. 5 ml of the anthrone reagent were added to 0.5 ml of the sample (about 100 micrograms per ml), (MARSDEN and ÖSTLING, personal communication).

After thorough mixing the tubes were warmed in boiling water for exactly 10 minutes and then cooled immediately in running cold water. The absorbance at 620 mμ was then measured with a Coleman Model 4 Spectrophotometer. In 10 determinations of the same sample the standard deviation was ±1.81%.

The blank solution (chapter V, A, b and c) contained very little anthrone-positive substance (corresponding to 0–8 μg hexose).

C. Determination of radioactive isotopes

a) *Gamma spectrometry*

The gamma spectrometer used in both influx and efflux experiments had a $1\frac{3}{4}''$ NaI(Tl) well-type crystal. A photo-multiplier (type RCA 6655) was found not to alter its amplification when the impulse frequency was changed (Öbrink and Ulfendahl, 1959). The high voltage supply (Tracerlab Precision High Voltage Supply) had a long-term stability of 0.02% per 24 hours or better. The linear amplifier (Tracerlab RLA-1) had good "non over-load" properties (complete recovery after a pulse 100 times too large was obtained within 6–10 microsec). Two pulse height analysers (Tracerlab RLA-5S) were used. The input voltage to the spectrometer was provided from a stabiliser (228 V ±0.2%).

The efficiency of this spectrometer system was about 10% with the window wide open.

The energy spectrum for the isotopes used was recorded as according to the method described by Öbrink and Ulfendahl (1959).

Both influx and efflux experiments make great demands on the gamma spectrometer. In an influx experiment the activity during a measuring period is very high, and in addition the radioactivity between the measuring periods is between 100 times and 1000 times greater than during the measuring period itself. During the influx experiments the radioactive analysis began only about 50 milliseconds after the radioactivity in the well crystal had been very high (see Fig. 23, p. 47). For such analyses a very good long- and short-term stability is required and also a linear response up to about 10,000 cps. Further the amplification must be constant and completely independent of the incoming pulse frequency. It was not permissible for the apparatus to have either a short- or long-term memory.

Analysis of a dilution series of samples of Cs^{137} (Fig. 16) with a wide open energy channel gives a good example of the linearity of the apparatus for incoming pulses (analysis in both energy channels gave the same result, but only one of these is shown in the figure). As is evident from Fig. 16 the coincidence losses between 0 and 10,000 cps were negligible.

Fig. 17 shows the gamma energy spectrum for Cs^{137} (sample 2^9 in Fig. 16), which was obtained by scanning with an energy channel of 0.004 MeV. By analysing a series of dilutions (the same series as in Fig. 16)

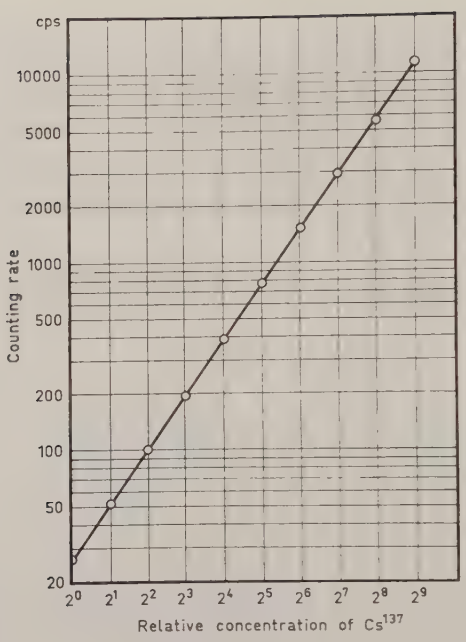

FIG. 16. Analysis of a dilution series of Cs^{137} (channel width 0–1.2 MeV).

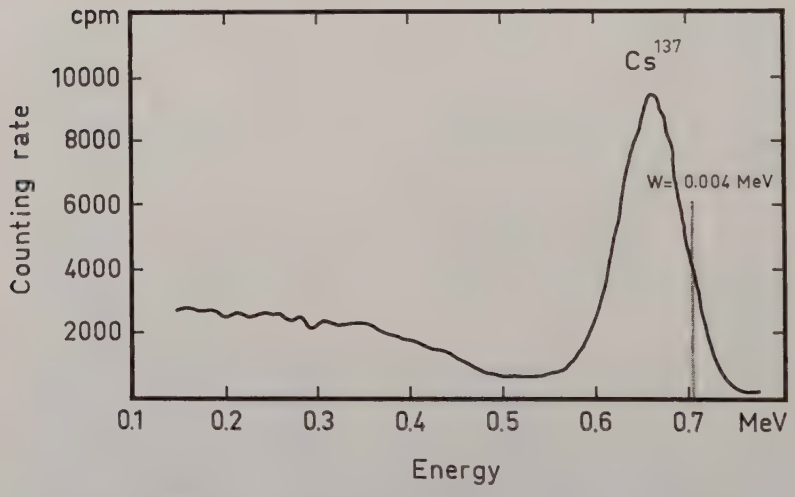

FIG. 17. Spectrum of Cs^{137} obtained by scanning with a channel width corresponding to 0.004 MeV ("line" in figure).

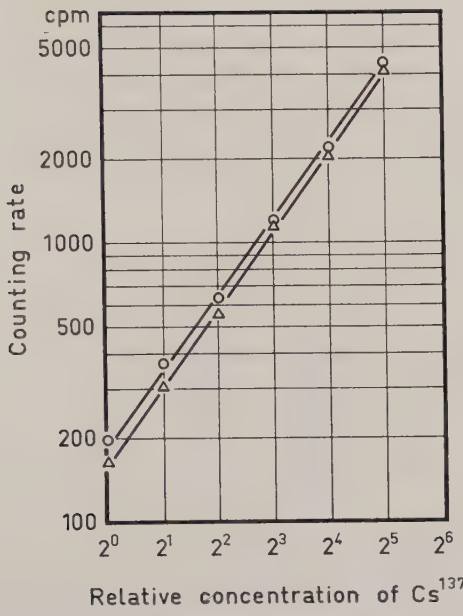

FIG. 18. Analysis of a dilution series of Cs^{137} with a narrow window positioned at the steepest part of the Cs^{137} photopeak, i.e. at the line *W* in Fig. 17 (circles, channel I). The other line (triangles) gives the results when the window was set at an energy level slightly higher (channel II). A linear relationship was obtained in both cases.

with a narrow window (the 0.004 MeV line in Fig. 17) on the steep slope of the Cs^{137} photo-peak the results shown in Fig. 18 were obtained. From these results it can be concluded that the amplification of the apparatus did not change up to an impulse frequency of 10,000 cps (the relative concentration of $Cs^{137} = 2^5$ in Fig. 18 corresponds to an input impulse frequency of about 10,000 cps).

The error in radioactive analysis arises from both the statistical error (the radioactive disintegration) and instability of the apparatus. The statistical error depends on the number of counts recorded (see for example SCHMEISER, 1961): If n is the number of recorded impulses (Poisson distribution) the statistical error in this determination is $\pm\sqrt{n}$, i.e. ± 1 S.D. $= \pm\sqrt{n}$ (variation coefficient, $v_{stat} = \pm\sqrt{n}/n \times 100\,\%$).

When a sample of Cs^{137} (2^5, Fig. 18) was counted repeatedly with a narrow energy channel situated as in Fig. 17 the coefficient of variation for 50 measurements was $v_{method} = \pm 0.25\,\%$ (DANIELSON and SJÖSTRAND, 1964). This is in fact the same value as the expected v_{stat} corresponding to ± 1 S.D. and it can therefore be concluded that the stability of the apparatus was very good, and that the analysis error for an isotope approached

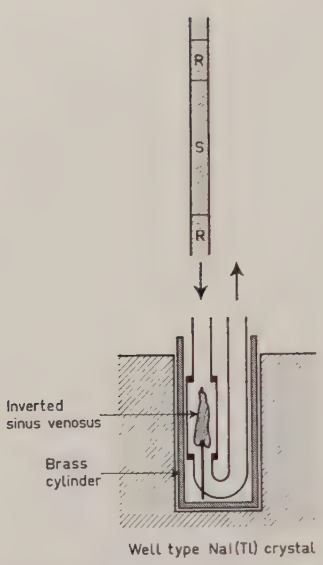

FIG. 19. Drawing of an isolated, inverted sinus venosus in the U-tube which formed the perfusion chamber in an influx experiment (DMM). The whole chamber is mounted in the well of the NaI(Tl) crystal. The alternate Ringer (*R*) and silicone oil (*S*) periods are shown diagrammatically above.

that dependent on the variations in the radioactive disintegration process alone. In the influx experiments a broad energy channel was always used (wide open window) and it can therefore be estimated that the error on analysis of a single isotope was approximately $\pm\sqrt{n}$.

1) *Determination of* Na^{24}, K^{42} *and* Br^{82} *in influx experiments, the direct monitoring method (DMM)*

In the influx experiments the perfusion chamber (see chapter III) was placed in the well of the crystal as shown in Fig. 19, so that good geometrical conditions were obtained. The crystal was mounted in a lead collimator. For the analysis of Na^{24} and K^{42} the perfusion chamber was surrounded by a brass sheath (3000 mg/cm^2). For the analysis of Br^{82} the brass sheath was replaced by one of perspex with the same dimensions. During the influx experiments therefore the preparation always occupied the same place in the well crystal, so that identical geometrical conditions were obtained. The brass sheath was calculated to absorb all beta particles with an energy lower than 4.0 MeV, without giving any significant "Brehmsstrahlung". Similarly it was calculated that the beta particles from Br^{82} should be stopped by the perspex sheath and the aluminium housing of the crystal. The preparation itself was 18–30 mm from the

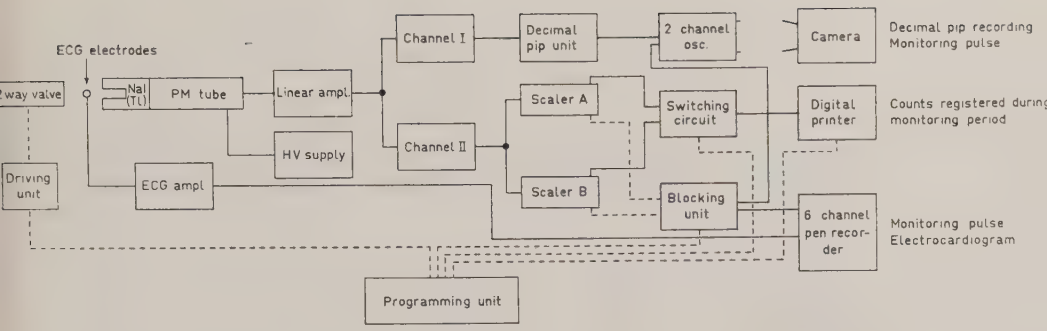

FIG. 20. Block diagram of the recording system used in an influx experiment with the direct monitoring method (DMM). For further details see text.

bottom of the well, in which position the relative counting rate was 97–94 % of the maximal obtainable if the tracer had been placed in the bottom of the well.

The radioactivity in the influx experiments was measured in two ways, as shown in the block diagram of Fig. 20. The pulses from one of the pulse height analysers (channel I) were fed into a decimal-pip unit (DANIELSON, ÖBRINK, SJÖSTRAND and WALLER, 1963). The pulses from the decimal-pip unit were fed into one channel of a two-channel oscilloscope (Tektronix type 502 Dual Beam), and the oscilloscope screen was photographed continually. A decimal-pip recording is shown in Fig. 23 (p. 47). The film speed was known, and therefore the radioactive counting rate during short time periods could be calculated with great accuracy.

This decimal-pip method is however rather time consuming, and another less laborious method was used for the analysis of the silicone oil perfusion periods during the long influx experiments. A digital recording method, as shown diagrammatically in Fig. 20, was used instead (PERSSON and SJÖSTRAND, 1964). Impulses from the second pulse height analyser (channel II) were fed into a common input for two electronic scalers (scalers A and B in the figure).[1] Scaler A or scaler B can be read alternately by a digital printer[2], which can make three readings in one second. The programming unit which controls the two-way valve which gives the alternate Ringer-silicone oil flow in the perfusion system in the influx studies controls also scalers A and B and the reading of these scalers by the digital printer. A monitoring pulse (DC), indicating when scalers A and B are counting, is recorded on a separate channel of the Grass Model 5 Polygraph (also used for continual ECG recording), and serves

[1] Transistorized scalers with Decade Counter Tubes type ELESTA EZ 10 B, see "ELESTA technical communications", No. 18, April 1962, ELESTA electronics Ltd., Switzerland.

[2] Kienzle Digital Printer type D1E, Kienzle Apparate GmbH, West Germany.

TABLE 3. *Simultaneous determination of radioactivity with both "decimal-pip" and digital printer systems.*

Monitoring time (t_m), msec	Counts recorded by printer system	Counts recorded by "decimal-pip" system	Cps according to printer system	Cps according to "decimal-pip" system
1315	320	320	243	243
1230	310	310	252	252
1225	343	330	280	269
1200	317	310	264	258
1185	272	270	230	228
1160	288	280	248	241
1175	306	310	260	264
1160	311	310	268	267
1160	269	260	232	224
1160	301	300	259	259
1125	357	350	317	311
1130	288	280	255	248
1140	307	300	269	263
1140	263	260	231	228
1120	306	310	273	277
1130	283	280	250	248
1140	254	250	223	219
			Mean: 256	Mean: 253

Simultaneous determination of radioactivity with both analysis systems of Fig. 20 ("decimal-pip" and digital printer systems). The monitoring time (t_m) was the same for both systems. The values shown correspond to the first 17 analysis points in the experiment illustrated in Fig. 27.

to indicate the analysis time (paper speed 100 mm/sec, see Fig. 23, p. 47). After each scaler has been read off by the digital printer it is immediately re-set to zero. The two scalers work alternately; while one is being read off the other is counting. The mean counting rate during the whole analysis period can thus be estimated.

The monitoring pulse was used to operate the digital printer while camera recording of the decimal-pip traces was made continuously. It seemed possible that the start and stop counting used by the digital printer system might give variations in the calculated counting rate, since the start and stop method might not be electronically stable. Table 3 illustrates a comparison between the results obtained with simultaneous determination of the counting rate by the decimal-pip and direct digital printer systems. As is evident from the table any differences are insignificant. The values shown in the table are the first 17 analyses from an experiment which is illustrated in Fig. 27 (p. 62).

In order to estimate the error of the method for the two analysis systems the dilution series of Cs^{137} used in the experiment illustrated in Fig. 16 (p. 36) were used (channels I and II: 0–1.2 MeV). The analysis periods were 1000 msec long and the duration could be accurately determined both from the Grass recorder and from the photographic recording (paper and film speeds 100 mm/sec), together with the monitoring pulse signals (50 analyses per min). 30–40 determinations were made for each sample of the dilution series. Up to a counting rate of about 10,000 cps the same coefficient of variation as expected from the random character of the radioactive disintegrations was obtained both for the decimal-pip and digital printer recording, i.e. the coefficient of variation $v_{stat} = v_{method} = \pm\sqrt{n}/n \times 100\,\%$.

Specific activity is defined as Curies per g of the element. In this paper, however, the radioactivity per equivalent concentration, i.e. kcps/μmole will be used, and this will be denoted as the equivalent specific activity (ESA); (kcps = 10^3 cps).

2. *Determination of Na^{24} and Br^{82} in mixtures*

The determination of the isotopes in such a mixture was performed as described by Öbrink and Ulfendahl (1959). To minimize the error wide energy channels were used so that a large number of impulses could be obtained in a short time. By selecting the channel boundaries in suitable parts of the gamma energy spectrum, it is possible to minimize effects of instability of the apparatus (cf. Danielson and Sjöstrand, 1964). For the analysis of Na^{24} and Br^{82} the channel settings were such that in channel I all energies below 4.0 MeV, and in channel II energies between 1.0 and 4.0 MeV were recorded. The results shown are in all cases the activities calculated for channel I. By frequent quotient determinations (each 5th sample was a standard) any drifts in the channels could be corrected. The accuracy of analysis in the determination of Na^{24} and Br^{82} is given for each experiment (see Danielson and Sjöstrand, 1964). In no sample did the volume exceed that which gave less than 99–100 % relative counting activity. In this way the recovery was constant for both isotopes. In all cases a correction for radioactive decay during the analysis time was made. The values given for the equivalent specific activities (ESA) for both isotopes in the soaking solution are corrected for decay to a constant starting time.

b) *Determination of S^{35} by liquid scintillation counting*

The determination of S^{35} was done in a Packard Liquid Scintillation Counter type TRI-CARB model 314 EX.

0.1 ml of sample (aqueous solution) was added to 3 ml of absolute alcohol in a plastic counting vial. 10 ml of scintillation fluid (1 litre toluol, 5 g PPO, 0.3 g dimethyl POPOP, as described by Hayes, 1962) were added. Since the alcohol and scintillation fluid were added with automatic syringe pipettes any errors so produced must have been very small.

D. Special methods

a) *Determination of the velocity of flow in the perfusion system*

1) *Determination of the linear mean velocity of flow for continual Ringer or silicone oil perfusion*

In these measurements the perfusion system and perfusion chamber were set up as described for the influx experiments (chapter III). The perfusion pressure was about 735 mm Hg (over-pressure), (Fig. 11, p. 28). The volume of perfusant passing the perfusion chamber in a minute was determined, and from the data of the perfusion chamber's dimensions the velocity could be calculated. The velocities shown in Fig. 14 (p. 31) are for Ringer and silicone oil flows calculated for the incoming tube (diameter 1.7 mm) to that part of the perfusion chamber where the preparation was mounted.

2) *Determination of the Ringer-silicone oil phase boundary velocity*

The Ringer-silicone phase boundary can be described as an acceleration–deceleration system and it would therefore be expected to have a lower speed than either Ringer or silicone oil alone. The Ringer solution is a volume conductor while silicone oil can be regarded as an insulator. The system shown in Fig. 21 was therefore used for determining the velocity of the passage of the phase boundary across the perfusion chamber.

Points *FW 1* and *FW 2* are equidistant from the centre of the preparation in the perfusion chamber. At each point a pair of silver wires (distance apart 2 mm) were inserted.[1] The resistance between the silver wires at points *FW 1* and *FW 2* was different for Ringer and silicone oil. This was recorded through a Grass 5Pl DC preamplifier and Grass Model 5 Polygraph. Since the distance between *FW 1* and *FW 2* (100 mm) was known, the velocity of the Ringer-silicone oil phase boundary could be calculated.

b) *Determination of the conduction velocity of the preparation*

The method is illustrated in Fig. 15 (see chapter IV, A, c, 2, suction electrode technique). This method allowed recording of action potentials in positions b_1 and b_2. The preparation was stimulated at the upper end with a rectangular pulse between two silver-silver-chloride electrodes (not drawn in the figure). The time difference between the action potentials recorded at the two sites AP 1 and AP 2 respectively could be determined. Assuming linear propagation in the preparation, the minimal conduction velocity for the distance b_1–b_2 (6.0 mm) could be determined.

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[1] The distance between *outlet S or R* in the rapidly acting two-way valve (Fig. 12, p. 29) and *FW 1* was 85 mm; the distance between *FW 2* and the *air inlet* (Figs. 11, 22, p. 28 and p. 46 resp.) was 160 mm.

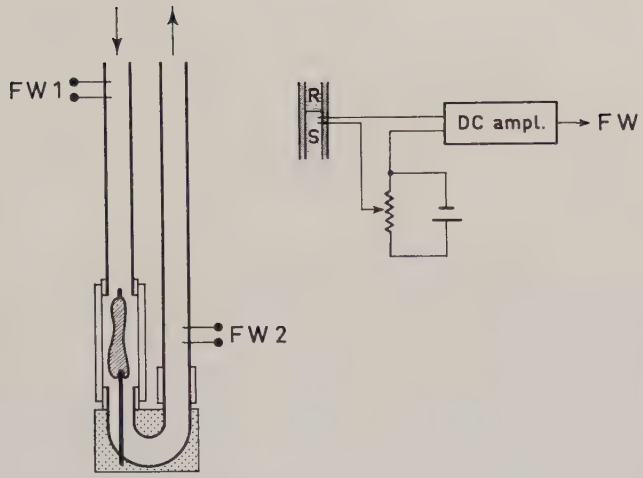

FIG. 21. System used for determination of the linear velocity of the Ringer-silicone oil phase boundary between points *FW* 1 and *FW* 2 which are equidistant from the tissue preparation. See text for further details.

E. Solutions

Solution I (*normal Ringer solution*): 6.5 g NaCl (111.2 mM), 0.2 g KCl (2.68 mM), 0.2 g $CaCl_2$ (1.80 mM), 0.1 g $NaHCO_3$ (1.19 mM) and 1 g glucose (5.55 mM) per litre H_2O (pH = 7.2).

Solution II: In the experiments where Br^{82} were studied part of the NaCl was substituted by NaBr. This Ringer solution contained 4.8 g NaCl (83.0 mM), 2.9 g NaBr (28.2 mM), 0.2 g KCl (2.68 mM), 0.2 g $CaCl_2$ (1.80 mM), 0.1 g $NaHCO_3$ (1.19 mM) and 1 g glucose (5.55 mM) per litre H_2O (pH corrected to 7.2).

Solution III: In the experiments where the different spaces of the preparation were studied the test substance was added to either solution I or II (without glucose).

a) 5% dextran Ringer (R_D), solution I without glucose, containing 50 g of dextran 250 per litre ($\bar{M}_W = 267{,}000$).

b) 5% ficoll Ringer (R_F), solution I without glucose, containing 50 g of ficoll per litre ($\bar{M}_W = 100{,}000$).

c) 2% raffinose Ringer (R_R), solutions I or II without glucose, containing 20 g (34 mM) of raffinose per litre ($M = 594$).

d) 2.5% sucrose Ringer (R_S), solution I without glucose, containing 25 g (73 mM) of sucrose per litre ($M = 342$).

Solution IV: In the time-lag studies a Ringer solution containing 44 mM K^+ per litre was obtained by substituting NaCl in solution I with equimolar quantities of KCl.

Solution V: In the efflux experiments made with $S^{35}O_4$ a Ringer solution which also contained SO_4 was used. This Ringer solution contained 5.7 g NaCl (97.5 mM), 1.4 g NaBr (13.6 mM), 0.2 g KCl (2.68 mM), 0.2 g $CaCl_2$ (1.80 mM), 0.1 g $NaHCO_3$ (1.19 mM), 0.1 g Na_2SO_4 (1.00 mM) and 1 g glucose (5.55 mM) per litre H_2O (pH corrected to 7.2).

No correction was made for the colloid osmotic pressure in the Ringer solutions either for the absence of plasma proteins or the presence of high polymers.

The pH of the Ringer solutions was always corrected to pH = 7.2 as there is presumably a marked pH effect on the electrolyte distribution (for K^+ and Cl^- in skeletal muscle cf. Brooks and Hutter, 1964).

Radioactive Ringer solution was obtained by adding $Na^{24}Cl$, $K^{42}Cl$, NH_4Br^{82} and $Na_2S^{35}O_4$ to the solution. Na^{24} and K^{42} were obtained by neutron irradiation of $NaHCO_3$ and K_2CO_3, which were then converted to the chlorides by titrating with HCl. Br^{82} was obtained by neutron irradiation of NH_4Br and dissolved after irradiation in distilled water.[1] When the irradiated material was added the Ringer solutions were corrected so that the molarities of the radioactive solutions I, II, III and V had the same values as given above. S^{35} was obtained in the form of carrier-free $Na_2S^{35}O_4$ in aqueous solution.[2] The calculated equivalent specific activity (ESA) is given in each experiment.

Both the Ringer solution and silicone oil were oxygenated before addition to the perfusion system (without CO_2).

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[1] The isotopes were obtained from AB Atomenergi, Stockholm, Sweden.

[2] The isotopes were obtained from The Radiochemical Centre, Amersham, England.

V. *Procedure*

A. Influx methods

Two types of influx methods were used. In one the preparation was perfused with an alternate Ringer-silicone oil flow with analysis of the preparation's radioactivity during the silicone oil period (see chapter III for the perfusion system and chapter IV for the direct monitoring method). The second influx method was more conventional (see for example WILDE, 1945). The preparation was suspended in a loading solution containing a test substance with vibration stirring, and after a certain time transferred to a bath of inactive Ringer solution. From the analysis of the washing solution the amount of test substance taken up could be calculated.

a) *Direct monitoring method (DMM)*

After the preparation had been mounted in the perfusion chamber (see Fig. 22, direct monitoring method), it was perfused with inactive Ringer solution for about 30 min (see preparation of the toad heart, p. 16), the ECG being recorded continuously during this period. If the preparation had a constant spontaneous frequency and the form of the ECG remained unaltered during this 30-minute period, the experiment was then continued if the position of the preparation had remained satisfactory during the preliminary perfusion. The programming unit was now adjusted so that counting (start and stop order to the digital printer system, see Fig. 20, p. 39) only took place while the preparation lay in the silicone oil phase. This was accomplished with the two pairs of resistance electrodes which had been used to determine the velocity of the silicone Ringer phase boundary (chapter IV, D, a, 2). In each experiment the length of the Ringer perfusion period is given as t_r (usually about 100 msec) that of the silicone oil perfusion t_s (≈ 1200 msec) and the duration of the radioactive measuring period (monitoring time) t_m (≈ 1000 msec). The next stage of the experiment was the replacement of those parts of the U-tube

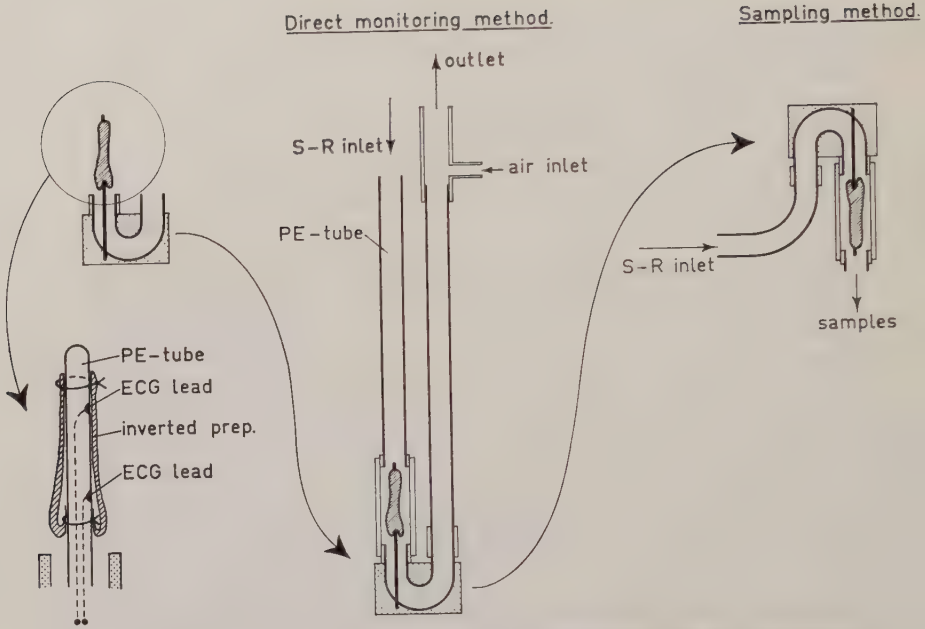

FIG. 22. The arrangements of the perfusion chamber used for the direct monitoring (DMM) and sampling (SM) methods. On the left is shown an inverted sinus venosus mounted on a polyethylene ECG catheter (*PE*-tube) attached to the U-tube. This is the unit which was used for both influx (DMM) and efflux (SM) studies. *S–R* inlet = silicone-Ringer inlet. The *air inlet* corresponds to that shown in Fig. 11 (p. 28).

system which contained the silver resistance electrodes by polyethylene tubes. It was now first necessary to wash out the inactive Ringer solution from the tube leading to the preparation chamber, so that the influx study should begin with perfusion by a solution of specific activity the same as that in the vessel *Ra*. The preparation was therefore perfused with silicone oil at a low velocity (low driving pressure) while the two-way valve in the *Ri-Ra system* (see Fig. 11, p. 28) was switched to "active". Thus active Ringer passes through to the rapid two-way valve and out through the *bypass outlet R* (Fig. 12, p. 29). The activity of the solution was monitored with a beta detector and a sample taken for later accurate determination of the activity per unit volume. The *bypass outlet R* was then closed, and the driving unit to the rapid two-way valve (see Fig. 20, p. 39) disconnected, while the background activity was determined with the digital printer system. During the following silicone oil perfusion, the perfusion

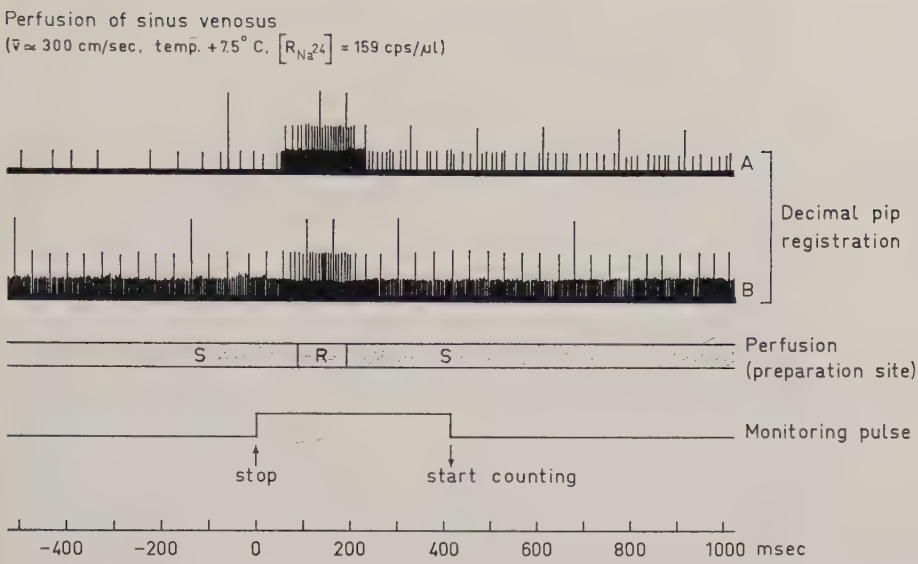

FIG. 23. "Decimal-pip" recording from two different periods in the influx experiment illustrated in Fig. 32 (p. II). *A* is the record of the oscilloscope screen showing the first active Ringer perfusion period (*r*1), *B* includes the record of the 76th active Ringer period (*r*76 in Fig. 32, curve I; $t_r + t_s = 143$ sec). The relative length of the active Ringer period (*R*) is shown schematically, flanked by silicone periods (*S*). It can be seen that this continuous recording reveals that the phase of increased activity precedes and lags behind a little the actual region of the active Ringer solution. As is shown the monitoring pulse stops recording by the digital printer both during the active Ringer period and immediately before and after it.

pressure was raised to about 735 mm Hg (over-pressure), and then the alternate perfusion with radioactive Ringer solution together with analysis of the activity during the silicone oil period was started. ECG and the monitoring pulse were recorded on the Grass recorder, while the decimal pip recording and monitoring pulse were photographed on the oscilloscope screen (cf. Fig. 23), which as a rule was only filmed during certain parts of the experiment. At the end of the experiment a sample of the active Ringer solution was taken via *bypass outlet R*.

b) *Soaking experiments with vibration stirring*

The preparation was mounted, as described, on the ECG catheter and was mounted in the perfusion chamber as for influx experiments. It was perfused for 30 min with inactive Ringer solution, after which

the preparation was perfused with silicone oil at full perfusion pressure ($\bar{v} \approx 300$ cm/sec) for 3 sec. The ECG catheter together with the preparation was then removed from the perfusion chamber and mounted on a stand. It was then dipped in "active" Ringer solution (active in this sense means that the Ringer solution contained the test molecules or isotopes). The vessel containing the active solution had a volume of 1 ml and was attached to the vibrating shank of a small magnetic vibrator. The active solution was oxygenated immediately before addition to the tube, and in those cases where the preparation was soaked for longer than 5 minutes, this was exchanged for freshly oxygenated Ringer solution each fifth minute. After a certain time the preparation was then quickly remounted for perfusion with silicone oil and perfused at a mean velocity of about 300 cm/sec for 3 seconds. This short silicone oil period washed away the greater part of the Ringer solution which had been carried over with the preparation which was then again removed from the chamber and washed in small vibration-stirred tubes containing inactive Ringer solution. The washing time was always at least $1\frac{1}{2}$ times the soaking time, but was never less than 10 minutes. The volume of inactive solution used in the "washing-off" tubes was about 0.5 ml (the exact volume was determined by weighing). The inactive washing solutions were also oxygenated and changed for new solution every fifth minute. The amount of test solute which had accumulated in the preparation was calculated by measuring the concentration in the pooled solution from all the washing tubes used. In order to check that all activity had been washed out of a preparation, it was in some cases verified that the concentration of the test species was zero in the last washing-out solution. The treatment in the Ringer baths was at room temperature, and the ECG was followed during the whole experiment. The washing-off phase with silicone oil was performed at $+5°C$ for 3 sec. This temperature was used in order to obtain similar surface layers around the preparation, as in the influx experiments made with the direct monitoring method. Since certain substances normally present in cardiac muscle give a positive anthrone reaction (carbohydrate determination), the blank solution for the anthrone determinations was obtained by washing-out the preparation in inactive Ringer solution for 10 minutes subsequent to a 10 minutes' soaking period in inactive solution.

c) *Perfusion experiments with washing-out of the activity in a static solution bath*

The preparation was mounted in the chamber as in the direct monitoring method and the procedure up to the washing through with and sampling of the active Ringer solution was the same as described in section a (direct monitoring method). The programming unit (Fig. 20, p. 39) was then adjusted for the selected Ringer perfusion time. The perfusion pressure was raised during the silicone oil perfusion to a value of about 735 mm Hg (over-pressure). The Ringer solution was then perfused for the selected period and this was followed by silicone oil perfusion ($\bar{v} \approx 300$ cm/sec) for 3 seconds. The preparation was then quickly removed from the perfusion chamber and washed in inactive Ringer at room temperature as described under section b. When necessary the blank for the anthrone method was also taken in the same way as described previously.

B. Efflux methods

a) *Sampling method (SM)*

The preparation was mounted on the ECG catheter in the chamber and perfused with inactive Ringer solution for 30 minutes; its viability was assessed as described previously under section A, a (DMM). The preparation was then removed from the chamber and loaded with the isotope to be studied by soaking for a certain time in an oxygenated and vibration-stirred bath of active Ringer solution (room temperature). The solution was renewed each tenth minute. The ECG was recorded continuously during the loading period. A sample of the last active Ringer solution was taken for analysis. The preparation was then returned to the chamber but this time mounted as for an efflux experiment as shown in Fig. 22 (p. 46, sampling method). It was then washed with silicone oil and the ECG and frequency of spontaneous beating were observed. When it was judged from the ECG that the preparation was in thermal equilibrium with the perfusion solution, inactive Ringer solution was perfused through the chamber and collected in test tubes mounted in a fraction collector operating at two changes per second. Each time the sample tube was changed this was recorded on one of the channels of the Grass Polygraph recorder (paper speed 50 mm/sec).

VI. *Some Influx Experiments; the Calculations and the Graphical Representation of Such Experiments*

As discussed previously (chapter IV, C, a, 1, direct monitoring method), the error of the method in the radioactive analysis in the systems used depends almost entirely on the number of impulses recorded. Since the monitoring time (t_m) was of the order of 900–1400 milliseconds, it was necessary if the counting error was to be small, to have a high radioactivity during the period of analysis. This means that the radioactive Ringer solution should therefore have a high specific activity. In influx studies, therefore, only isotopes where the decay gave a large percentage of gamma radiation were suitable.

The ions which from a biological point of view are of greatest interest are Na^+, K^+, Cl^- and Ca^{++}. Na^{24} can be obtained with a very high specific activity, and has two gamma peaks (1.37 and 2.76 MeV), both of which are obtained in 100 % of disintegrations. In the case of potassium the only suitable isotope available until recently is K^{42}; this has a gamma peak at 1.53 MeV, but only 18 % of disintegrations give this energy. With the irradiation of normal potassium an insufficient specific activity of K^{42} is obtained even with high neutron fluxes. By irradiating enriched K^{41} or through the irradiation of normal potassium with very high neutron fluxes, the specific activity of K^{42} can be increased, but this is very expensive. Because therefore of the relatively low specific activity of K^{42} only a few experiments have been made with this isotope (see Fig. 31, p. 69).

Cl^{36} is only a beta emitter, so it is not suitable for an influx experiment. Cl^{38} is from many points of view unsuitable. It has a short half-life and a beta energy of 4.81 MeV (53 %), which makes it somewhat of a health hazard. Under the assumption that Br^- and Cl^- are distributed in the same way in heart tissue (Lamb, 1961; Hutter, 1961; Burrows and Lamb, 1962), experiments have been made with Br^{82}. According to Danielson (1964) the Br^- and I^- ions distribute

in the same way as the Cl^- ions in the sinus venosus, atrium and ventricle of the toad. Br^{82} can be obtained with high specific activity and has eight gamma peaks between 0.55 and 1.47 MeV. Of the Ca isotopes, Ca^{45} has no gamma emission, while Ca^{47}, although a gamma emitter, was rather too expensive for an influx experiment.

An influx experiment with Na^{24}

Fig. 24 illustrates an influx experiment with Na^{24} on an isolated sinus venosus. The preparation had an ECG which showed that for each heart cycle there were two electrical activities (cf. Fig. 9, p. 24). It was also observed that the preparation had two mechanical activities. The upper part contracted just before the lower part. Two influx experiments were made on this preparation, of which the first is illustrated in the upper part of the figure. The radioactivity counted during a silicone oil period has been recorded as though it occurred just at the end of the foregoing Ringer perfusion period. The radioactivity counted increased with time during alternate active Ringer and silicone oil perfusion.

After the first influx experiment was finished, all radioactivity was removed from the preparation by perfusion with inactive Ringer solution for about 30 minutes.

A second influx experiment was then made with the same active Ringer solution as previously, and the result is illustrated in the lower part of Fig. 24. The sinus venosus beat spontaneously for about 26–27 seconds, when a much stronger ECG complex than had previously been observed appeared; subsequently no further electrical activity was recorded. It is evident from the figure that the radioactivity during the first 20 seconds increased at about the same or a little lower rate than in the first experiment. After 22–23 seconds the rate of increase with time rapidly lessened. The reason for this sudden cardiac arrest is not clear. The spontaneous activity, however, returned when at the end of the experiment the preparation was removed from the chamber and bathed in Ringer solution at room temperature. It therefore seems possible that an undue temperature sensitivity may have been responsible for the cessation of spontaneous cardiac activity. If the radioactivity recorded is representative of that taken up during an influx experiment by the sinus venosus, there thus seems to be a connection between the electrical and/or mechanical activity of the

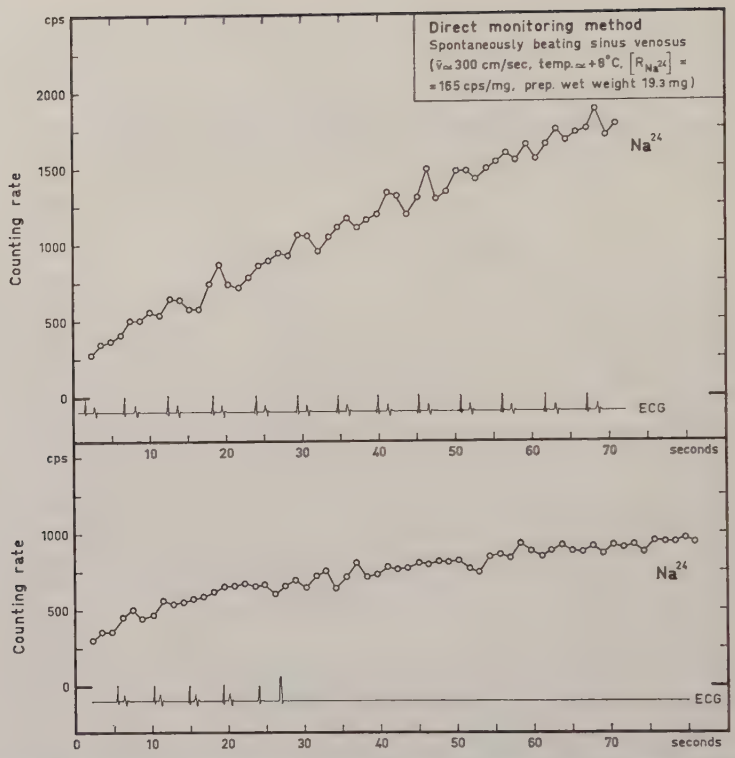

FIG. 24. Two influx experiments with Na^{24} on an inverted, spontaneously beating sinus venosus perfused alternately with active Ringer solution and silicone oil. The radioactivity was measured during the later period (DMM). The ECG showed that for each heart cycle there was a double electrical event (see text). The radioactivities recorded have been arbitrarily assigned to the time point of the end of the preceding Ringer perfusion period. The upper part of the figure shows the first influx experiment and the lower part the second experiment (about 30 minutes after the first) during which the electrical activity ceased. The surface film (O_R) was calculated as equivalent to 1.64 mg of active Ringer solution, and the "trapped volume" as 0.66 mg; $t_r = 105$ msec, $t_s = 1195$ msec, $t_m = 928$ msec and $t_{reqv} = 400$ msec. The mean cycle calculated for the first experiment is shown in Fig. 47 (p. 117).

preparation and the rate of uptake of Na^{24}. HAAS, GLITSCH and TRAUTWEIN (1963) have shown that Na^{24} efflux from an isolated auricle of the frog is greater in a beating than in a non-beating preparation (cf. introduction).

An influx experiment with Br^{82}

Such an experiment is illustrated in Fig. 25 (p. I), where it is evident from the electrocardiogram that the sinus venosus beat with a very

regular frequency during the whole experiment. The electrical activity appeared to arise from a single pace-maker (ECG), and it was observed visually that the mechanical activity in each cycle was single. The radioactivity recorded during the monitoring period (silicone oil perfusion) has been plotted, as previously, as though it occurred at the end of the foregoing Ringer perfusion period. It is evident that the radioactivity increased with time during the experiment.

Trends in the data or only background noise?

It is evident from the Na^{24} experiment, and even more so from the Br^{82} experiment, that if there are any real variations associated with single cardiac cycles, they are confused by other variations, such as "random fluctuation noise" in the perfusion system, statistical counting errors and instabilities in the recording system. It cannot therefore be expected that radioactivity recorded from each single cardiac cycle will give precisely the same picture. The analysis period used, i.e. 1.2–1.3 sec is relatively long in relation to the biological process being studied. Thus the influx method from this viewpoint can be regarded as having a rather low resolution. In order to ascertain the underlying biological pattern, it is therefore desirable to calculate the variations in counting activity for a *"mean cycle"*.

"Continuous averaging" is a problem which has been treated, *inter alia*, in connection with the analysis of the electroencephalogram. For this purpose a number of different mathematical and graphical methods or harmonic Fourier analysis have been used. More recently computers often called CATs (computers of average transients) have been used for solving these problems. Their task has been to calculate the mean value of, or summate, responses as they occur in real time, and to accumulate this information. The CAT technique has been reviewed by Garry (1961). The CAT is, however, not directly applicable to these experiments, and for this reason a graphical method has been used to obtain the "mean cycle". This method will be called the *"computing of average transients"*, *c-a-t*.

Essential requirements for c-a-t

The primary assumption in a *c-a-t* is that the biological activity curve is a continuous function (a *c-a-t* is in fact a smoothing or curve fitting procedure for the oscillatory component), and that the obser-

vations are frequent and of short duration compared to the biological oscillations. The radioactivity measurements in the actual inflow experiments are of relatively long duration and hence few in number in each cycle, so that it may appear questionable as to whether they can be used to deduce a continuous type of biological activity curve.

In the analysis described here the number of observations per cycle is small (in Fig. 25 ≈ 7) and therefore *the observations from a number of cycles are used to derive the probable activity curve for an 'average single cycle'*. There is therefore the added absolute requirement that during the actual influx experiment the relationship between the "*mean influx*" during the Ringer and silicone periods must remain constant for the whole experiment. It is assumed, of course, that the biological processes are essentially the same in each cycle. Further, variations in the perfusion or analysis systems must be of a purely random nature.

The c-a-t procedure in a Br^{82} influx experiment

The experiment illustrated in Fig. 25 serves as an example of the determination of the "*mean cycle*" by the *c-a-t* procedure in an influx experiment. As is evident from Figs. 24 (p. 52) and 25 (p. I) the Na^{24} and Br^{82} activities appear to consist of at least two components:

1. an exponential influx curve (*DC component*), on which are superimposed
2. oscillations (*AC component*).

1. *DC component.* It will be shown in chapter VII that the requirements stated above for the *c-a-t* are approximately valid.

The first stage is to establish a "*mean trend*" for the increase in activity as a reference or *base line*. Even if the biological significance of this line is not established with certainty it seems justifiable for mathematical reasons as a seemingly suitable regression line for all the points. The equation for the line which best fits the observations will be called the *mean trend*, and can be derived with a curve fitting procedure as described by Lewis (1960). The general form of the equation is first assumed; with the influx experiments it is of the general form $y_t = a(1 - e^{-kt}) + y_{t=0}$, where y_t is the recorded activity in cps at time t seconds after the beginning of the experiment, and a and k are constants.

Fig. 25 (p. I) shows the mean activity calculated for each heart cycle (solid circles) and it can be seen that they lie scattered closely about the curve (calculated from all the points—empty triangles). For the *c-a-t* procedure in the other influx experiments the *mean trend* base line has therefore been constructed in a simpler way from the mean values for each cycle rather than from all the individual measurements. The lines so obtained are all loci of the type of exponential function given above.

2. *AC component.* The single heart cycle is arbitrarily defined as beginning 2 seconds before depolarization and is in the experiment Fig. 25 7.4 seconds long, i.e. taking depolarization as $t=0$ from -2 to $+5.4$ seconds. This is illustrated in Fig. 26 which is an analysis of the results of the experiment of Fig. 25; the figure also shows the first half of the "next cycle" which is, of course, merely a repetition of the first cycle. *All radioactive measurements are expressed as deviations from the mean trend line, and their temporal position in the cycle was determined graphically* (as mentioned previously each measurement is treated as though it occurred instantaneously at the end of the Ringer period preceding the silicone period during which the measurement was made).

The distribution of the radioactive measurements at different time intervals within each heart cycle is relatively uneven.[1] *The cardiac cycle was arbitrarily divided into 200 msec intervals and the radioactivity measurements were classified graphically into these classes,* which are designated by their central values. The numbers in each class varied randomly from 4 to 17, the arithmetic mean being 10.6. Thus the number of observations (N) in class $t=400$ msec (301–500 msec) was much less than in class $t=1400$ msec (1301–1500 msec). When N is small such a point is statistically less reliable than with a large value of N, and *a curve drawn by interpolating such values is likely to be more uncertain in some intervals than in others.* Both random noise (instability) and statistical counting error are very important

[1] The measurements were made at regular time intervals and the heart beat with a relatively constant frequency. There were thus two systems with regular frequencies. Under certain conditions such systems can exhibit a constant phase pattern with respect to each other, i.e. behave as though they were locked. This would mean that the measurements were repeatedly confined to the same parts of the successive cardiac cycles. This was not observed in these experiments, however.

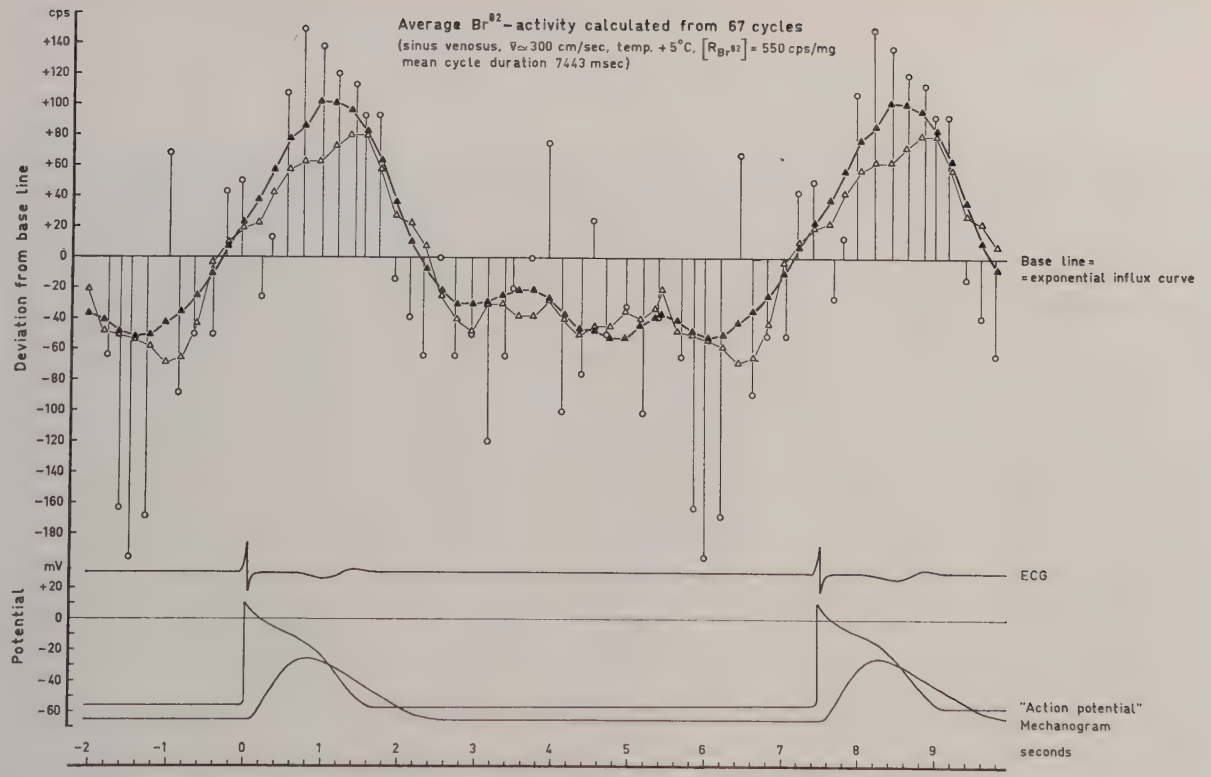

FIG. 26. A mean cycle for Br^{82} calculated by the *c-a-t* method for the experiment of Fig. 25. The "base line" corresponds to the mean trend line in Fig. 25 (curve I). The ECG was recorded in the experiment while the action potential and mechanogram traces are drawn from other experiments of the same type where they and the ECG were recorded (chapter II, c). The circles represent the median values for the measured values classified into 200 msec intervals ('original value method'). The open triangles are the medians and the solid triangles the arithmetic means of 67 interpolation values for each 200 msec interval ('intercept–interpolation method'). The standard deviations calculated for the 'original value method' and the arithmetic means of the 'interpolation method' are given on p. 57.

The maximal error of the mean radioactivity curve with respect to time (i.e. to electromechanical cycle) is $(t_r + t_s) = (100 + 1104) = 1204$ msec to the left (cf. p. 83 and p. 120). The wet weight of the preparation was 16.1 mg, O_R was calculated as 1.4 mg and the trapped volume as 0.7 mg. Perfusion and analysis programme (DMM): $t_r = 100$ msec, $t_s = 1104$ msec, $t_m = 930$ msec. Active Ringer solution ESA 19.5 kcps Br^{82}/μ mole Br (solution II). (kcps = 10^3 cps)

here. It seems therefore better to use another type of interpolation method. *In each heart cycle the points of observed radioactivity have been joined by straight lines, and the values of the intercepts of these lines with the central t class values which they cross have been recorded.* Thus for each *t* class there are as many intercept values as cardiac cycles measured during the experiment (67 in the experiment of Fig. 26), independently of how many actual radioactive measurements fell within that *t* class interval. There was of course quite wide variation in the intercept values for any particular class, but extreme variations showed a tendency to cancel out each other. The result should perhaps be a more certain assessment of the average value for each *t* value than with mere interpolation from the mean values of the observations themselves.

An important question is therefore as to how the observed values of the radioactivity should be treated, i.e. classified into time intervals and the mean class values used directly or subjected to a more complicated interpolation procedure as described above. Further, in taking averages should the arithmetic mean or the median be used?

Fig. 26 shows three out of the four possible methods. The '*original value method*' (open circles and vertical lines); these are the median activities in each *t* class (200 msec). The results of the '*intercept–interpolation method*' are also illustrated in the figure; the averages of 67 intercept values in each 200 msec interval are calculated from the *arithmetic means* (solid triangles) and the *medians* (open triangles). As is evident from the figure all three methods give essentially similar pictures. The area enclosed by the curve above the line is also approximately equal to that below the line, which should be expected when a mean cycle about a base line is determined.

The points of the '*original value method*' in Fig. 26 are the median values of the observations from all the heart cycles classified into 200 msec intervals. The number of values in each class interval ranged from 4 to 17. It is evident that the average values exhibit irregular and large positive and negative deviations, although the general trend is clear. The standard deviation of the mean in the intervals with the greatest number of observations (8 intervals had 14–17 observations) was, on the average, ± 7.7 (cps). The corresponding value for the same eight intervals in the '*intercept-interpolation method*' (67 points in each case) was ± 6.5. The difference is not significant (i.e. with $N = 16$, and $N = 67$, respectively, it is not even

as great as the mean error of the difference), but with a lower value of N extreme values would have more influence. With a standard deviation of about 7.0 the mean error with for example only 6 observations ($N=6$) will become more important. It is therefore not surprising that a direct use of the average of the observations in each class interval ('original value method') gives a much less smooth result than with 'intercept–interpolation method'. The latter may therefore give a clearer picture of the activity pattern.

Fig. 47 (p. 117) *shows a similar treatment of the results of a* Na^{24} *influx experiment.* Only median values were used for both the original value and intercept–interpolation methods, which show fairly good agreement if allowance is made for the irregularities of the former method. In Fig. 48 (p. 118), which illustrates another experiment, the arithmetic means have been used in calculating the points by the two methods. *From these results it is evident that in calculating the "mean cycle" the interpolation method is more satisfactory. It must, however, be considered which average should be used, the median or arithmetic mean.* In general the median is more suitable if N is small, and extreme values are more likely to be disturbing and produce asymmetric distributions. If there are no or very few asymmetric extreme values the arithmetic mean can equally well be used even with a small number of observations. Since it seems reasonable to presume that the biological flux pattern is a continuous funtion of time this means that *large variations in the recorded radioactivity can be expected to result from such factors as the length of the analysis period in relation to the period of the biological curve and the temporal position of the analysis period in relation to the biological events.*

Assuming that this argument is correct the calculation of *mean values from the interpolated values should be used only if N is relatively large.* Only then can it be assumed that extreme values resulting from "random noise" in the system will be cancelled out. "Biological" extreme values should then be revealed by the averaging of the intercept–interpolations. *When N is small the median should be used* in order to minimize the effect of extreme values which may be of "random noise" character.

It should be further pointed out that the 'intercept–interpolation method' does itself modify the pattern of radioactivity. The amplitude of fluctuations from the base line will be reduced somewhat but the variations around the mean will not be essentially altered (cf. Figs. 26, 47

and 48—'original value method' and 'intercept–interpolation method' points). If the radioactive measurements are made over shorter time intervals this effect will be reduced. Asymmetry around the t axis (base line) can also result in *small phase shifts* occurring when the 'intercept–interpolation method' is used.

VII. *Some Methodological Problems of the Influx Experiments*

Some of the most important problems associated with the results discussed in chapter VI must now be appraised.

A. The magnitude of the extraneous or "background" activity is important in the influx experiments. This activity is partly due to imperfect shielding from active Ringer solution in the incoming and return perfusion lines. A further source of activity extraneous to the tissue preparation is the "trapped activity" in the perfusion chamber itself; this was measured in a sham experiment without any tissue in the chamber.

B. For physical reasons it can be expected that the tissue preparation will be surrounded by a thin surface film of Ringer solution during the silicone oil perfusion period. The thickness of this layer and whether it remains constant during one silicone oil period and from period to period are important.

C. If there is a significant surface layer whether the rates of increase of radioactivity in the tissue during Ringer and silicone oil perfusion are the same. During Ringer perfusion the radioactivity "source" is virtually infinite while in the silicone period it is finite and not very large. The increases therefore during the two periods must be expected to be *different functions* of time, but it is important that their *mean values* have a constant ratio during the whole experiment (when the perfusion intervals are held constant).

D. The volume(s) in the tissue preparation which are most nearly equilibrated with radioactivity during the experiment.

E. The time lag between contact with the perfusing solution (Ringer) and entry of radioactivity into the tissue with the DMM (transport across a surface layer).

F. The effect of the mechanical contraction on the surface layer (or extracellular space).

Experimentally it is simpler to approach problems B and C with an object whose structure is much better defined than in a biological preparation (e.g. cellophane or an ion exchanger). As the biological problem in the present experiments has been the primary aim, it seemed more important to attempt to appraise these matters in relation to the actual heart preparation used, as they are very important in the analysis and interpretation of the results of both influx and efflux experiments. A more systematic study of, for example, an ion exchanger has not yet been made but seems desirable.

The methods used for studying problems A–F will be described below, and *the most important methodological problems concerning the results will be discussed; the question of their biological significance will be deferred to general discussion.* Point F will be mentioned very briefly.

A. The background activity and "trapped activity"

Sham "influx" experiments for Na^{24} and Br^{82} were made with an empty perfusion chamber; in all other respects the technique was as described in chapter V, A, a (direct monitoring method). The result for a sham Na^{24} "influx" experiment is shown in Fig. 27, where the background was determined before the start of an alternate Ringer-silicone oil perfusion ($t_r = 100.4$ msec, $t_s = 1320$ msec, $t_m = 1160$ msec, see definition in chapter V, A, a). It can be seen that after the first active Ringer period the radioactivity lies around a level which subsequently remained constant at a mean value of 60 cps above background. From the known counting activity of the active Ringer solution (cps/μl, 1 μl $\approx$ 1 mg), the trapped activity can be estimated as equivalent to 0.42 mg of the active Ringer solution. The line best fitting the observations was calculated by BARTLETT's (1949) method. The coefficient of variation was $v_{\text{method}} = \pm 8.7$ %. Analysis of a Cs^{137} sample in the same analysis system and method (see chapter IV, C, a, 1) gave a coefficient of variation $v_{\text{stat.}} = \pm 5.9$ % ($v_{\text{stat.}} = \pm\sqrt{n}/n \times 100$ %). In a similar experiment with Br^{82} the activity of the Ringer solution was 174 cps/mg ($t_r = 100.4$ msec, $t_s = 1320$ msec and $t_m = 1001$ msec). The trapped activity corresponded to 0.90 mg active Ringer solution ($v_{\text{method}} = \pm 8.9$ % and $v_{\text{stat.}} = \pm 5.3$ %); in this experiment also the chamber activity reached a 'constant' level after the first perfusion period.

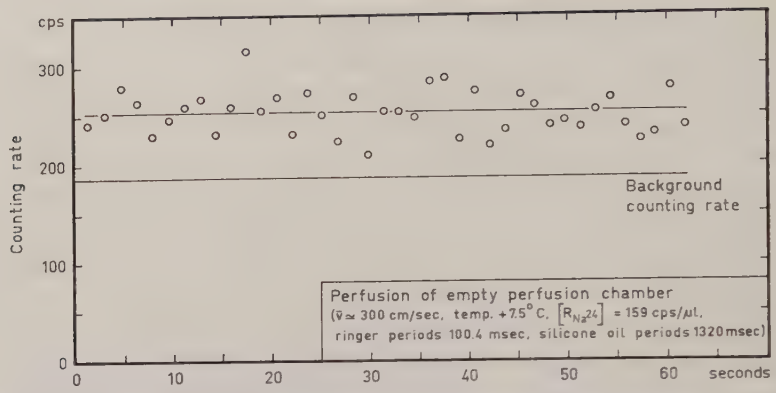

FIG. 27. Perfusion of an empty chamber with active Ringer solution (DMM). The first 17 analyses are also shown in Table 3. $v_{method} = \pm 8.7$ %, $v_{stat} = \pm 5.9$ % (cf. text).

The *"trapped volume"* will be assumed to be equivalent to a mean value of 0.7 mg Ringer solution in those cases where it was not measured. Whether the difference between the Na^{24} and Br^{82} values is random or not is not known, but practically it seems justifiable to use the average value.

The *background activity* was 186 cps in the Na^{24} experiment corresponding to 1.17 mg of the active solution, and with Br^{82} 215 cps, corresponding to 1.21 mg.

Summary: The background activities in sham "influx" experiments were equivalent to about 1.2 mg active Ringer solution, while the "trapped activity" remaining in "sight" of the NaI(Tl) crystal corresponded to 0.7 mg. *During sham perfusion with alternate active Ringer solution and silicone oil as described for DMM* (chapter V, A, a) *the radioactivity varied randomly around a mean value* (cf. Fig. 27) *where the coefficient of variation* (v_{method}) *was only a few per cent greater than the statistical counting error* (± 1 S.D.) *for the mean number of counts recorded* (total counts including background).

B. The surface layer of the heart preparation during Ringer and silicone oil perfusion

As mentioned earlier both the Ringer and silicone oil flows are turbulent. In the turbulent Ringer flow good contact between the perfusant and the tissue can be expected, even if an "unstirred layer"

cannot be avoided. The thickness of the unstirred layer is difficult to estimate theoretically in the system used (see Jost, 1952, p. 78) but in physical studies of flows in the vicinity of surfaces Bock (1963) concluded that "unstirred layers" of the order of 0.5–1.0 μ might exist for "Newtonian liquids". Copley, Scott Blair, Baléa and Staple (1960) showed, that this order of magnitude also applied to the "plasmatic zone" in blood vessels of the Chinese Hamster. Schulman and Teorell (1938) and Manegold and Kalauch (1939) gave values for unstirred layers of the order of 10–30 μ. Since silicone oil is strongly hydrophobic it may be expected that during its passage through the perfusion chamber the tissue preparation will be enveloped by an aqueous surface film (O_R) consisting of Ringer solution, notwithstanding the turbulence of the silicone oil stream. There will thus be an interface between the external surface film of aqueous solution and the main silicone oil phase. The surface aqueous phase under these conditions is probably larger than the "unstirred layer" in Ringer perfusion. During silicone perfusion the aqueous surface film may however undergo a certain agitation due to pressure variations resulting from turbulence of the passing silicone oil stream. Such pressure variations may also, of course, be transmitted to the tissue preparation itself.

The volume of the surface film O_R during silicone oil perfusion

Studies of this parameter require water soluble and non-ionic (low affinity for tissue proteins) test solutes. Ideally the test substance should be specific for the surface film, i.e. either excluded from or only very slowly penetrating into the other regions of the tissue. (No such ideal substance exists and tests must be made on different substances with the aim of being able to extrapolate the results for the case of the ideal solute.) For a direct analysis with DMM a substance, labelled with a gamma-emitting isotope obtainable in high specific activity, which does not alter the normal physical properties of the Ringer solution, is required. Suitable "gamma labelled" substances were not available, so indirect methods were used instead.

Four "non-electrolytes" were used, all of which can be determined with high accuracy by an anthrone method. These were dextran[1] ($M_w = 267{,}000$), ficoll ($\overline{M}_w = 100{,}000$), raffinose ($M = 594$) and sucrose ($M = 342$). Dextran is uncharged polyglucose and takes the configu-

[1] Dextran and ficoll were kindly supplied by AB Pharmacia, Uppsala, Sweden.

ration of a loose random coil in solution (NEELY, 1960). Ficoll, a polysucrose, is a neutral but more compact molecule in solution; its solutions have a much lower viscosity than those of dextran. The molecular sizes of both dextran[1] and ficoll are such that during the short "soaking time" diffusion could not account for any significant penetration of the extracellular space. Raffinose and sucrose have often been used to determine extracellular space but according to BOZLER (1959, 1961*a*) sucrose penetrated also the intracellular compartment of the frog sartoris muscle (soaking time = 2 hours). The concentrations used were 5 g % for dextran and ficoll, 2 g % for raffinose and 2.5 g % for sucrose (*w*/*v*). The concentrations were held as low as practicable in order to reduce the osmotic effects on the tissue. In order to compare the figures for all four substances determined with the anthrone method, in the experiment of Fig. 28 the values for raffinose have been multiplied by 2.5, and those for sucrose by 2. The solubility of dextran, ficoll, raffinose and sucrose in silicone oil is virtually zero.[2] After soaking, the tissues were placed in the perfusion chamber and washed with either a 3 or 10 second period of silicone oil (linear velocity 300 cm/sec, see chapter V, A, b) which was saturated with the test substance. The preparations were then removed and the amounts of the carbohydrates taken up in the soaking bath were estimated; there were no significant differences, i.e. the 3 second silicone period was as effective as the 10 second period.

To obtain more reliable determinations with this method it seemed desirable that the amount of substance taken up should be larger, so the experiments were carried out on isolated atria of toads.

Fig. 28 shows an experiment where a spontaneously beating atrium was bathed for different periods in dextran-Ringer (R_D), ficoll-Ringer (R_F), raffinose-Ringer (R_R) or sucrose-Ringer (R_S) with vibration stirring according to the method described in chapter V, A, b. This is

[1] The so-called mean Stokes-Einstein radius of this dextran fraction is estimated as 70 Å (GROTTE, 1956).

[2] The distribution coefficient for dextran between silicone oil and Ringer solution was determined by shaking together aliquots of 5% dextran, Ringer solution and silicone oil (MS 200, 2 cS) for 5 min. After the two phases had separated half of the silicone oil phase was removed, mixed with an aliquot of normal Ringer solution and shaken for 5 minutes. The dextran concentration in this second Ringer solution was then determined by the anthrone method and the distribution coefficient of (Ringer): (silicone oil) was calculated from a duplicate determination as 667:1.

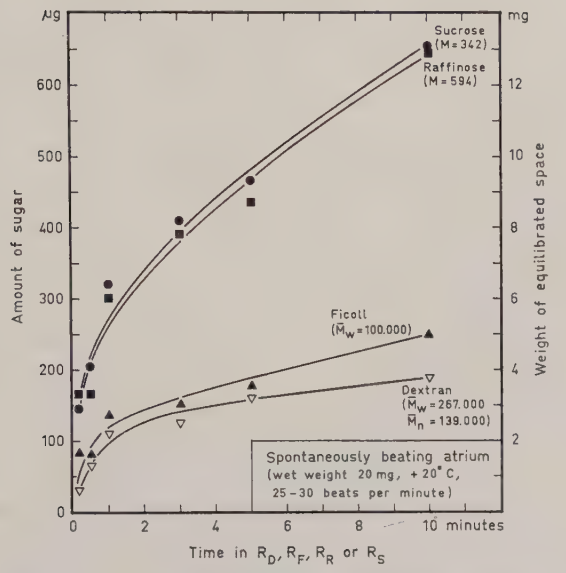

FIG. 28. Soaking experiment with vibration stirring as described in chapter V, A, b (see text).

one of two experiments, both of which showed similar results. By assuming that the test molecule equilibrates with an accessible compartment to the same concentration as in the active Ringer solution, an *equilibration volume* can be estimated. The figure shows the relative rates of penetration of the four substances into the preparation. *It appears probable that all four substances equilibrated initially with a space of 2–3 mg in weight. A subsequent distribution took place into what appears to be a second compartment.* This distribution shows a relationship to the decreasing molecular weight (or increasing diffusion coefficient). *It seems reasonable to suppose that the initial equilibration space is a surface film to which all substances have a very rapid access, and the second compartment is an extracellular space or even perhaps partially intracellular* (BOZLER, 1961*a*).

Fig. 29 shows the results of experiments made according to the method described in chapter V, A, c, where the points in the figure are mean values of the results from several preparations (see figure text). *The figure can be regarded as representing to a large extent the experimental conditions during an actual influx experiment by the DMM, and shows that equilibration of the surface layer takes place in less than*

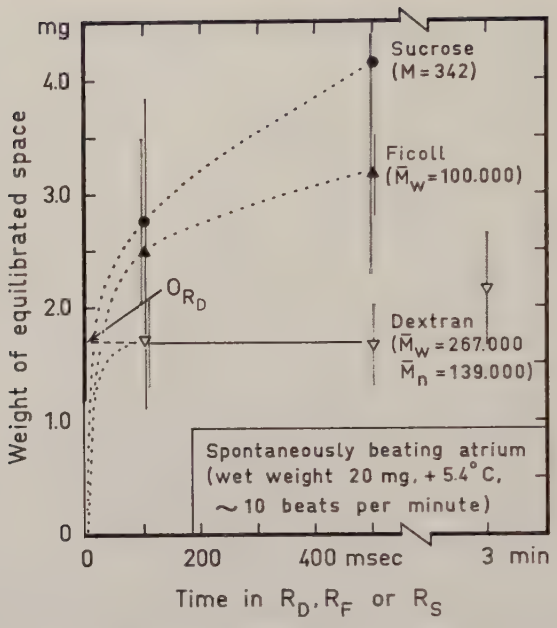

FIG. 29. A perfusion experiment with washing out in a static bath on spontaneously beating atria with dextran (R_D), ficoll (R_F) and sucrose (R_S). The procedure was as described in chapter V, A, c and the perfusion times 100 msec, 500 msec and 3 minutes. For R_D 3 atria were used, for R_F 4 atria and for R_S 2 atria. The number of measurements at different perfusion times were:

100 msec 21 (R_D), 18 (R_F) 9 (R_S)
500 msec 14 (R_D), 21 (R_F) 9 (R_S)
3 minutes 8 (R_D)

The standard errors are drawn in the figure. The amount of test substance was corrected in all cases to a preparation wet weight of 20 mg. The external surface film (O_{R_D}) was with dextran equivalent to 1.7 mg of the Ringer solution (8.5% of the wet weight).

100 msec for all substances (dextran, ficoll, sucrose). *There was no penetration of dextran into the second compartment during a 500 msec long perfusion period* (cf. also the mean values for dextran-Ringer solution for a 3-minute period). *Dextran thus seems a suitable indicator substance for the surface layer*, at least under the perfusion conditions existing in the influx experiments (DMM, $t_r \approx 100$ msec). *This extra-cardiac surface layer* (O_{R_D} in the figure) *during the silicone oil perfusion should therefore for dextran have a volume corresponding to 1.7 mg of Ringer solution (8.5 % of the wet weight)*. It seems improbable that any interaction between dextran and the surface proteins of the preparation contributes to this to any significant extent, because

it is apparent from the figure that the minimum sucrose space is similar (there is no reason to suppose that sucrose should be absorbed to any significant extent). The amount of substance which would accumulate on the preparation assuming a complete monolayer was calculated to be less than the anthrone method error.

In order to assess correctly the results shown in Figs. 28 and 29, certain other factors must be taken into account. The presence of 5 % dextran in the Ringer solution greatly increases its viscosity, while ficoll, raffinose and sucrose in the concentrations used have an insignificant effect on the viscosity (solutions I and II). The colloid osmotic pressure in the dextran and ficoll-Ringer solutions can be neglected (ROWE, 1955, found that a 4.2 % dextran solution was isosmotic with 7 % serum proteins; at the concentration and with the molecular weight range of the fraction used here the osmotic pressure is virtually a function of the concentration). The osmotic effects of the oligosaccharides will be treated later (p. 87).

In the discussion which follows, the *extra-cardiac surface film* (O_R) *during silicone oil perfusion* has been assumed to have a size corresponding to that determined with dextran (O_{RD}), i.e. corresponding to *8.5 % of the wet weight of the preparation.*

"Unstirred layer" during Ringer perfusion

Since the equilibration at the surface during a dextran perfusion seems to require less than 100 msec (cf. Fig. 29), *it appears reasonable to assume that during the Ringer perfusion periods in the influx experiments* (*DMM*), *the surface film* O_R *acquires the same specific activity as that of the active Ringer solution.* The minimal surface area of the preparation (the external surface of an open cylinder) was used to calculate that the maximal thickness of the surface film during the silicone oil perfusion ($O_{RD} = 8.5$ % of the wet weight) was 25–30 μ (the minimal areas for the preparations used ranged from 69 to 71 mm^2). It is, however, improbable that the unstirred layer during Ringer perfusion had this thickness, because a complete equilibration of so thick a surface layer through pure diffusion in a free water phase is impossible for a molecule as large as dextran ($\overline{M}_W \approx 250{,}000$). It is more likely that in the Ringer perfusion period the unstirred layer was thinner since, as is evident from Fig. 29, the equilibration volume for sucrose after 100 msec Ringer perfusion is somewhat greater than for dextran. Assuming that the diffusion co-

efficient is the same as in the free aqueous phase, in 100 msec NaCl would attain equilibrium with a layer having a thickness of about 7μ (calculated as diffusion in a plane sheet, see CRANK, 1956). The amount of dextran which would be found in the corresponding volume (assuming the minimal area of the preparation) is of the same order as twice the error of the anthrone method. Sucrose and ficoll (Fig. 29, p. 66) equilibrate in a larger volume than dextran. It therefore seems likely that the *thickness of the unstirred layer during Ringer perfusion is from 0.5* (cf. p. 63) *to about* 7μ. In the influx experiments made according to the DMM, it was found that after the first active Ringer perfusion period there was a certain amount of the tracer (Na^{24}, K^{42}, Br^{82}) already taken up by the preparation.[1] *It does not therefore appear probable that the "unstirred layer" during the Ringer perfusion period is the rate-limiting step for the distribution of tracer between active solution and the preparation.* The distribution of tracer between the active solution and the tissue preparation is discussed under section C (cf. also section E).

The volume of the extra-cardiac surface film O_R during extended silicone oil perfusion

As is evident from the decimal-pip recording in Fig. 23 (p. 47) the boundary between active Ringer perfusion and silicone oil perfusion is, as far as the radioactive analyses are concerned, rather sharp. With the change from turbulent Ringer to turbulent silicone oil streams the intervening phase boundary passes the perfusion chamber quickly (cf. Fig. 13, p. 30). As soon as the phase boundary has passed, the counts recorded consist of 1) the background activity, 2) "trapped activity", 3) radioactivity in the preparation itself and 4) the radioactivity in the extra-cardiac surface film (O_R).

To study the radioactivity recorded in the system immediately after passage of the Ringer-silicone oil phase boundary, the experiments illustrated in Figs. 30 and 31 were performed. Sinus venosus from toad was used, in one case beating spontaneously, and in the other in a non-beating condition due to the addition of potassium to

[1] The experiment illustrated in Fig. 24 (p. 52) is an exception, because a calculation of the counting activity of the surface film O_R plus the "trapped activity" gave a value of 380 cps. A probable reason for this seems that the surface film and/or "trapped activity" in this experiment were smaller than calculated.

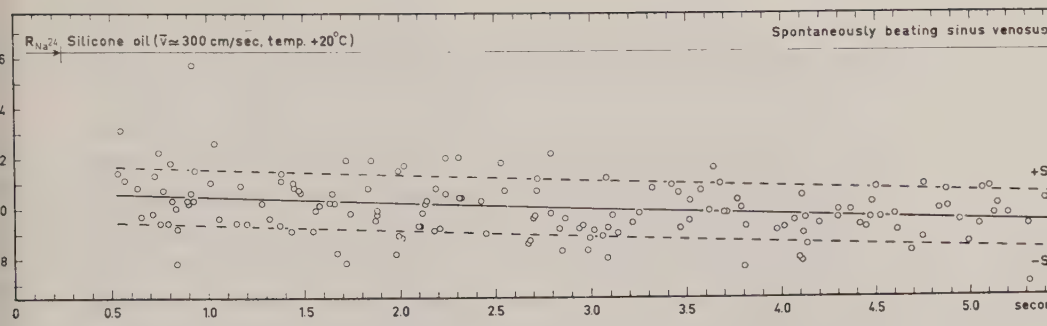

FIG. 30. The relative activities measured during the silicone oil periods in an influx experiment (Na^{24}) on a spontaneously beating sinus venosus. The activities recorded were estimated by the "decimal-pip" system (see text for further details).

the Ringer solution (about 20 mM K^+). The length of the Ringer perfusion period (t_r) was 200–300 msec, while that of the silicone oil was varied (up to 6 seconds). With the aid of the decimal-pip system the mean value for the counting rate during the whole silicone oil perfusion period and the time intervals between each 100 pip impulses was estimated. The counting rate for each 100 pip interval was plotted in relation to the mean counting rate for the whole period.[1]

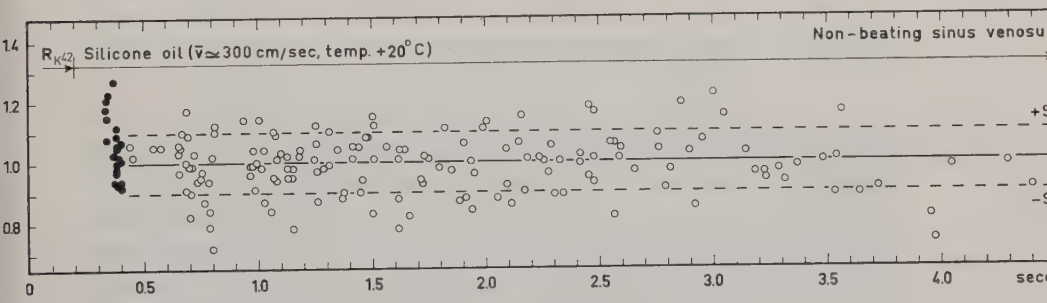

FIG. 31. The relative activities measured during the silicone oil periods in an influx experiment (K^{42}) on a non-beating sinus venosus. The solid circles represent analyses not included in the standard error of the estimate (see text for further details).

[1] Because of the limited thickness of the extra-cardiac surface film (O_R), the activity of Fig. 30 presumably does not truly decrease linearly with perfusion time, but it is probable that in a period as short as is illustrated in the figure, linearity can be approximately assumed. For this reason BARTLETT's (1949) method has been used to determine the regression line. The standard error of the estimate was calculated as $S = \pm\sqrt{\sum(x-\bar{x})^2/(N-2)}$.

In the experiments with a spontaneously beating sinus venosus, the relative counting rate decreased by 2 % per second for the experiment with Na^{24} (Fig. 30) and also for the experiment with K^{42} made on the same preparation. The standard error of the estimate was calculated for Na^{24} as $(S_{Na^{24}})_{method} = \pm 0.11$, for K^{42} as $(S_{K^{42}})_{method} = \pm 0.12$ (according to chapter IV, C, a, 1, a standard error of the estimate $S_{stat} = \pm 0.10$ could be expected). Fig. 31 shows the results for K^{42} in a non-beating sinus venosus. The regression line shows that the activity remained constant during the whole of the silicone oil perfusion; the standard error of the estimate being $(S_{K^{42}})_{method} = \pm 0.10$. It was observed that the counting activity during silicone oil perfusion did not always decrease in a spontaneously beating preparation. As was shown in an experiment at +8°C when the radioactivity (Na^{24}) was measured during a silicone oil perfusion period 21 seconds long, the counting rate was maintained during the whole of this perfusion time at the same level (cf. also Fig. 33, p. 72).

It was therefore evident in Figs. 30, 31 and 33 that *the counting activity during silicone oil perfusion was relatively constant during each individual experiment, with perfusion times of about 1 second* ($t_s \approx 1$ sec in the influx experiments by DMM).

Summary: It is concluded that *during Ringer perfusion the preparation is surrounded by an unstirred layer with a probable thickness of 0.5–7 μ.* During active Ringer perfusion it is improbable that the unstirred layer is the rate-limiting step as regards distribution of the tracer between the perfusion fluid and the preparation. *With change over to silicone oil perfusion an extra-cardiac surface film of active Ringer solution (O_R) remains after passage of the Ringer-silicone oil phase boundary. This surface film of aqueous solution has the same specific activity as the active Ringer solution at $t_s = 0$, and has probably a mean size of about 8.5 % of the wet weight of the preparation* (the maximal thickness 25–30 μ). A small part of the material of this surface film appears to be transported away during silicone oil perfusion, most probably by the turbulent silicone oil stream, which may tear off small drops of Ringer solution; the counting activity in the system thus decreases at a rate of between 0 and 2 % per second (some distribution of the tracer between the surface film of the preparation and the turbulent silicone oil stream is also possible). *To simplify the discussion the counting activity in the silicone oil perfusion period will*

henceforth be treated as though it were constant, because, as can be seen in Fig. 33 (p. 72), the reduction in radioactivity was relatively constant in each separate experiment. The sham influx experiments (p. 61) indicated that the *trapped activity varied randomly* from perfusion period to perfusion period. *Whether the volume of the extra-cardiac surface film O_R only exhibits random fluctuations from perfusion cycle to perfusion cycle is difficult to assess* (systematic fluctuations in the volume of this film resulting from the mechanical activity of the preparation cannot be excluded); see also summary, section C.

C. The uptake of tracer in the tissue of the preparation during Ringer and silicone oil perfusion

It was argued in the previous section that there are probably differences in the boundary processes for distribution of the tracer during Ringer and silicone oil perfusion. During active Ringer perfusion (t_r) the tracer can exchange between the solution and preparation; with silicone oil perfusion any exchange mainly occurs between the tissue and the extra-cardiac surface film (O_R). The exchange kinetics between the perfusing Ringer solution or the surface layer during the silicone oil period and the tissue may be considered as a case of diffusion through a thin film; the film thickness, however, is probably much greater in the latter cases. The system used in these flux experiments is basically similar to the material transfer problems encountered in ion exchange or chromatographic processes where there are two main transfer barriers, (1) the diffusion within the gel particle (gel diffusion kinetics) and (2) the transfer from outside solution to the gel phase (film diffusion kinetics). *Film diffusion becomes increasingly important as the solute concentration decreases and may become the rate limiting step.* These processes are discussed exhaustively by Helfferich (1959), (see also Teorell, 1953). The rate of uptake for the isotope studied will be expected to be higher during the active Ringer perfusion period, because during this time the preparation is washed continually by a solution with a constant specific activity, while during the silicone oil perfusion period the only source of external radioactivity is in the extra-cardiac surface film of limited volume and can be expected to diminish somewhat during the silicone oil perfusion, a condition which further complicates the issue. With more information about the distribution conditions during the differ-

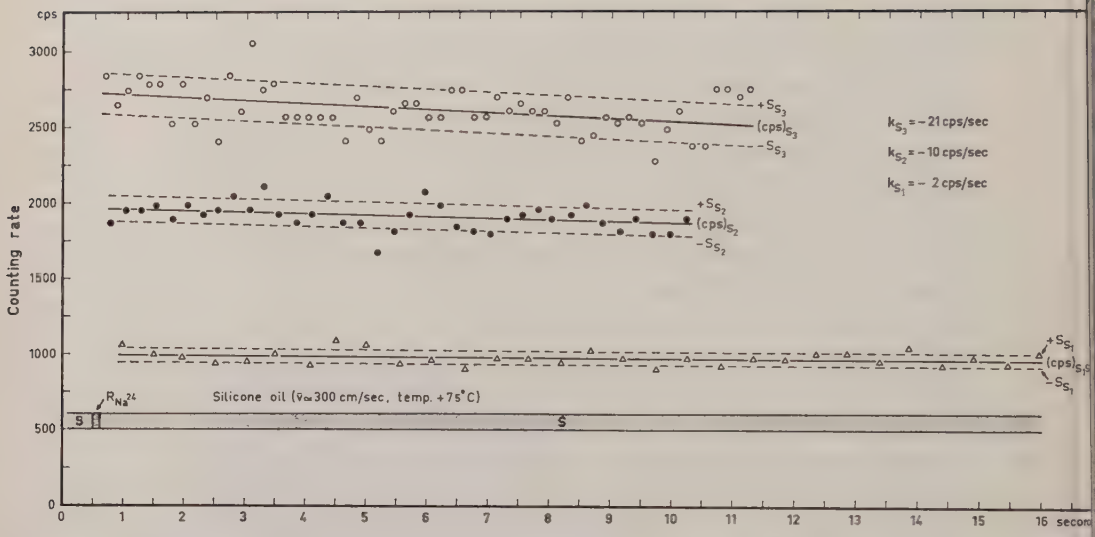

FIG. 33. The radioactivity in the system during the long silicone oil perfusion periods S_1, S_2 and S_3 of the experiment of Fig. 32. The activity was measured by the "decimal-pip" system (see text). The standard error of the estimate was calculated as:

$S_{S1} = \pm 46$ cps (43), $S_{S2} = \pm 86$ cps (84), $S_{S3} = \pm 136$ cps (115).
The figures in parentheses are the S_{stat} values. The error in the case of S_3 is large because of the slow film speed used in the recording (higher activity).

ent perfusion phases, it might be possible to make a correction in an influx experiment so that the real connection between the increase in activity of the preparation and the active Ringer perfusion could be estimated.

An influx experiment (DMM) interrupted with long periods of Ringer or silicone oil perfusion alone

Fig. 32 (p. II) illustrates an experiment made on a spontaneously beating sinus venosus with active Ringer solution containing Na^{24} ($[R_{Na^{24}}] = 159$ cps/mg). The preparation was perfused alternately with active Ringer solution and silicone oil at a temperature of $+7.5°C$ and a mean perfusion velocity of about 300 cm/sec ($t_r = 100.4$ msec, $t_s = 1320$ msec, $t_m = 1001$ msec). The radioactivities recorded have been plotted as though they occurred instantaneously at the end of the preceding perfusion period. The open circles of *curve I* in the figure are the successive means of three consecutive measurements (i.e.

each point is the mean of the points in the immediately preceding, the same, and the immediately succeeding measuring periods). This was done in order to smooth out fluctuations. The alternate Ringer-silicone cycles were interrupted by long Ringer or silicone oil perfusion periods (R_1, R_2, R_3, R_4 and S_1, S_2, S_3, S_4, S_5). Curve I thus describes the influx experiment. The increases in activity between *b–c*, *d–e*, *f–g* and *h–i* are those which occurred during the long perfusion periods with active Ringer solution (R_1, R_2, R_3 and R_4), and have been interpolated as linear functions of time.

Fig. 33 illustrates the activity in the long silicone oil periods (S_1, S_2, S_3) in the same experiment (the counting rate was calculated from the time required for a collection of 500 impulses in the decimal-pip system, cf. Figs. 30 and 31, p. 69). During the silicone period the activity decreases a little with time (see discussion in section B). The decrease occurred more rapidly during S_3 than S_2 or S_1 with values of 0.8 %, 0.5 % and 0.2 % per second respectively of the mean activity during the period. This can probably be explained by the specific activity in O_R decreasing more slowly during S_3 than during S_2 or S_1.

Calculation of the influx resulting from continual active Ringer perfusion

The time scale of *curve I* in Fig. 32 (p. II) is that of the actual experiment. *Curve II* has been plotted by including only the time periods of the Ringer perfusion ($t_r = 100.4$ msec) and neglecting the times of the silicone periods. The solid circles are the individual measurements made during the alternating silicone oil periods and lie in the intervals *ab*, *cd*, *ef* and *gh*. Each interval represents the total time for which the preparation was in the perfusing Ringer solution during that part of the experiment. The increases during these periods are faster than in the long pure Ringer periods (*bc*, *de*, *fg*, *hi*), which is interpreted as indicating that the time scale in these sections (*ab*, *cd*, *ef*, *gh*) is false. In these parts of the actual experiment the distribution of tracer to the tissue has also occurred during the silicone oil period from the extra-cardiac surface layer O_R. Assuming that the intervals *bc*, *de*, *fg* and *hi* represent the real conditions with pure long active Ringer perfusion, a "true" scale can then be calculated for *ab*, *cd*, *ef* and *gh*. The result of this is shown in *curve III*. The rate of uptake in the section *bc* of *curve II* is $(\Delta \text{ cps})_{bc}/t_{R_1}$, where $(\Delta \text{ cps})_{bc}$ is the in-

crease of activity during t_{R_1} where t_{R_1} = actual time of active Ringer perfusion. Hence for *de* we have $(\Delta \text{ cps})_{de}/t_{R_2}$ etc. During the interval *cd*, on the other hand, the actual distribution time for the tracer between the tissue and the active Ringer phase (active Ringer perfusion during t_r and the surface film O_R during t_s in each cycle) is equal to $\Sigma t_r + \Sigma t_s$; in curve II only Σt_r was used. For the construction of section *cd* of *curve III* the following expression was used:

$$\frac{(\Delta \text{ cps})_{cd}}{\sum t_r + \sum t_{r_{\text{eqv}}}} = \frac{\dfrac{(\Delta \text{ cps})_{bc}}{t_{R_1}} + \dfrac{(\Delta \text{ cps})_{de}}{t_{R_2}}}{2} \qquad (1)$$

In this way one can calculate the additional time required ($\Sigma t_{r_{\text{eqv}}}$) to adjust the distribution rate under this section of the curve (II) to the "true" value providing there were a constant specific activity around the preparation during this time. Curve III (Fig. 32) has been plotted by adjusting the relevant sections of curve II in this way (*ab*, *cd*, *ef* and *gh*).

From the uptake rates of periods *ab*, *cd*, *ef* and *gh* in curve I, a new curve (IV) has been drawn in which corrections have been made for the regions of the long Ringer (R) and silicone (S), where the alternate Ringer–silicone period pattern was interrupted. *Curve IV* thus represents the result that would have been obtained if the alternate perfusion periods t_r and t_s had not been interrupted.

Fig. 34 (p. II) *is a schematic representation of Fig. 32 and illustrates graphically the arguments presented above.* Curve IV, as in Fig. 32, represents the theoretical increase in activity as a function of time if the alternate Ringer–silicone oil perfusion had been maintained during the whole experiment (t_r/t_s = const.). Curve II is drawn schematically, while curve III is that calculated as in Fig. 32 for the approximately "true" relationship between the increase in activity of the preparation and time required if there had been continual perfusion with active Ringer solution (t_R). Sections *ab*, *cd*, *ef* and *gh* were calculated in this way. For example in section *cd* the activity increase during alternate perfusion ($\Sigma t_r + \Sigma t_s$) was 450 cps, with

$$\frac{t_r}{t_s} = \frac{\sum t_r}{\sum t_s} = \frac{100.4}{1320} = 0.076.$$

Now an increase by 450 cps would also occur with a perfusion period of Ringer alone, and the length of this period would be t_R and t_R =

$\Sigma t_r + \Sigma t_{r_{eqv}}$ (equation 1). During Σt_r the increase can be calculated as 80 cps (curve III). The remaining activity of 370 cps arose therefore in Σt_s by distribution of the tracer between the preparation and the surface film (O_R) during silicone oil perfusion. *As can be seen in the schematic construction the ratio* $(\Delta \text{ cps})_{\Sigma t_r}/(\Delta \text{ cps})_{\Sigma t_s} \approx$ *constant with the assumption that* t_r/t_s *is also constant during the whole experiment. This is a necessary requirement for a c-a-t* (see chapter VI, d).

The experiment illustrated in Figs. 32 and 34 is the only one of this type made but the results are so clear that they should aid in the understanding of the problems involved.

The mean concentration of Na^{24} *in the extra-cardiac surface film* (O_R) *during silicone oil perfusion*

The data of the experiment of Figs. 32 and 34 (p. II) can be used to calculate the mean concentration (= cps Na^{24} per mg of surface layer) of Na^{24} in O_R provided certain assumptions are made. The agitation which almost certainly occurs in this layer due to the adjacent turbulent silicone oil stream (cf. pp. 63, 68–71) may tend to create homogenety in the Na^{24} concentration.

Assuming that the volume of O_R is constant (cf. summary of section B) and that at $t_s = 0$ the specific activity in O_R is equal to that of the active Ringer solution (cf. p. 70) the following equation is valid (conservation of tracer):

$$(C_o)_1 \cdot V_o - (C_o)_2 \cdot V_o = (C_p)_2 \cdot V_p - (C_p)_1 \cdot V_p; \qquad (2)$$

where

V_o = volume of O_R (assumed to be 8.5 % of the wet weight),
V_p = volume of the heart preparation (density = 1),
$(C_o)_1$ = tracer concentration (cps/mg) in O_R at time $t_s = 0$,
$(C_o)_2$ = tracer concentration (cps/mg) in O_R at time t_s,
$(C_p)_1$ = tracer concentration (cps/mg) in the tissue at $t_s = 0$,
$(C_p)_2$ = tracer concentration (cps/mg) in the tissue at time t_s,

and

$$\underbrace{V_o \cdot [(C_o)_1 - (C_o)_2]}_{\text{amount of tracer leaving } O_R \text{ during } t_s \text{ msec of silicone oil perfusion.}} = \underbrace{V_p \cdot [(C_p)_2 - (C_p)_1]}_{\text{amount of tracer entering the preparation during } t_s \text{ msec of silicone oil perfusion.}} = \Delta \text{cps}_s; \qquad (3)$$

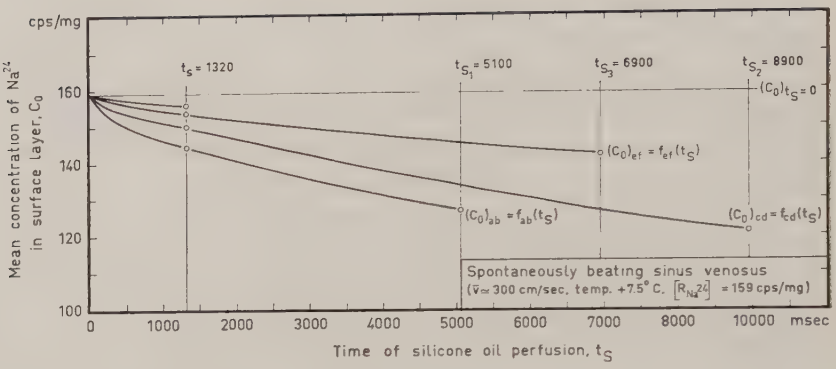

FIG. 35. Calculated mean concentration (C_0) of Na^{24} in the external surface film (O_R) surrounding the tissue preparation during silicone oil perfusion. The topmost curve corresponds to $(C_0)_{gh} = f_{gh}(t_s)$. The point for $(C_0)_{t_S}$ was not used since a large error might have been introduced because of the very small slope of the curve in this range. For further details see text.

Equation (3) is valid if both V_o and V_p are constant, and can be rewritten

$$(C_o)_2 = (C_o)_1 - \frac{\Delta \text{ cps}_s}{V_o}; \tag{4}$$

In this equation $(C_o)_1$ is the concentration of the active Ringer solution (cps/mg), $V_o = 8.5\,\%$ of the wet weight of the preparation (mg) and Δ cps$_s$ is its activity increase during silicone oil perfusion (cps). By determining $(C_o)_2$ in the intervals *ab*, *cd*, *ef* and *gh* and utilizing the single measurements made after the long silicone oil perfusion periods (S_1, S_2 and S_3) in the experiment of Fig. 32 (p. II), the mean concentration of Na^{24} in O_R was calculated; the results are given in Fig. 35.

In the experiment (Fig. 32), $(C_o)_1 = 159$ cps/mg, $V_o = 1.99\,\mu$l (= mg), Δ cps$_s$ can be obtained from the calculated increase in activity of the preparation during the intervals *ab*, *cd*, *ef*, *gh* as illustrated in Fig. 34 (p. II). $(\Delta \text{ cps})_{cd}$ for $t_s = 1320$ msec was calculated from the increase in activity during silicone oil perfusion [$(\Delta \text{ cps})_{\Sigma t_s} = 370$ cps] and the number of silicone oil periods ($N_{t_s} = 19.7$ during *cd*, curve I).[1] The value of $(C_o)_2$ is then calculated as for example in the interval *cd* for $t_s = 1320$ (cf. eqn. 4),

[1] The number of silicone oil perfusion periods 1320 msec long in the different intervals of curve I of Fig. 34 were, *ab* 21, *cd* 20, *ef* 38 and *gh* 46. The calculated increase in activity of the preparation during silicone oil perfusion for the corresponding intervals is shown in Fig. 34, $(\Delta \text{ cps})_{\Sigma t_s}$.

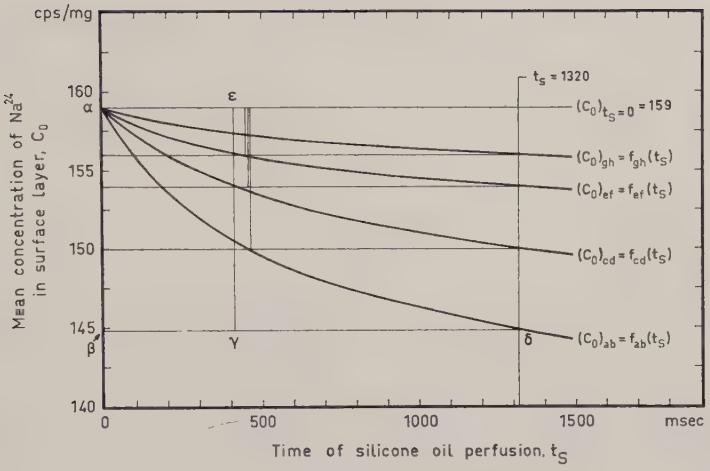

FIG. 36. Graphical construction of the mean concentrations (C_0) in the external surface film (O_R) during silicone perfusion between $t_s = 0$ msec and $t_s = 1320$ msec for different periods of the influx experiment illustrated in Figs. 32 and 34 (p. II). The t_{reqv} values ($\gamma - \beta$ in the figure) were for the intervals: *ab* 411 msec, *cd* 469 msec, *ef* 463 msec and *gh* 458 msec. The mean value for t_{reqv} was 450 msec.

$$(C_o)_{t_s=1320} = 159 - \frac{(\Delta \text{ cps})_{\Sigma t_s}}{N_{t_s} \cdot V_o} = 159 - \frac{370}{19.7 \cdot 1.99} = 150.$$

By using the measurements made immediately after the end of each long silicone oil perfusion period [S_1, S_2 and S_3 in Fig. 32; this is the radioactivity measured during the first ordinary silicone period (1320 msec) after the first active Ringer period (100.4 msec) after S_1, S_2 or S_3] estimates of Δ cps$_s$ were obtained. The values calculated are drawn in Fig. 35.

Fig. 36 is a graphical construction of the mean Na^{24} concentration in the layer O_R during silicone perfusion (V_o = constant). The ordinate also represents the amount of "tracer" which leaves O_R, and equation (4) can be written:

$$\Delta \text{ cps}_s = V_o \cdot [(C_o)_1 - (C_o)_2]. \qquad (4\,a)$$

It is assumed that for only a very short time after $t_s = 0$ the distribution in the tissue is at the same rate as with the active Ringer perfusion (t_r) and that the mean concentration of "tracer" in O_R at $t_s =$ 1320 msec can be obtained from the functions shown in Fig. 35. The

quantity of "tracer" which during 1320 msec of silicone oil perfusion (δ–β in the figure) entered the preparation in different periods of the influx experiment (*ab*, *cd*, *ef*, *gh*) can be expressed as the quantity of tracer which would enter during $t_{r_{eqv}}$ (in active Ringer) for the corresponding period of the experiment (γ–β), (cf. Fig. 34).

The uptake of Na^{24} in the tissue during active Ringer and silicone oil perfusion

Fig. 37 shows the calculated increase in activity of the tissue during Ringer and silicone oil periods at different intervals during the experiment (see Figs. 34 and 36). For section *ab* (Fig. 34) $t_r = 100.4$ msec (α in Fig. 37) the increase is 6.4 cps and for $t_s = 1320$ msec (γ in Fig. 37) the corresponding value during silicone oil perfusion 27.4 cps. The intercept β gives $t_{r_{eqv}}$ during interval *ab*.

Fig. 37 shows that the activity increases during Ringer and silicone oil perfusion follow different functions with respect to time. If these results are generally valid *the figure should serve as a "nomogram" for calculating the "true" time relations between increase of activity in the tissue and the active Ringer perfusion time for other influx experiments* where t_r/t_s has other values and where ions with approximately similar properties to Na have been used (cf. p. 88).

Reconstruction of an interval of an influx experiment

With regard to the arguments developed (Figs. 32–37) a *reconstruction of a small part of interval ab of Fig. 32 (p. II) will now be described.* Fig. 38 illustrates three Ringer-silicone oil perfusion cycles. Curves II, III and IV have been taken as linear over this short time period. The lower part of the left ordinate shows the total Na^{24} activity in the surface film (O_R) and the amount of "trapped activity" which has been assumed constant (cf. Fig. 27, p. 62). V_o and $(C_o)_{t_s=0}$ have also been taken as constant, i.e. $V_o \cdot (C_o)_{t_s=0} = 1.99 \times 159 = 316$ cps (background activity also assumed constant). The right ordinate gives the Na^{24} activity measured during silicone oil perfusion [zero = counting rate–background (186 cps)]. Cps_{s1}, cps_{s2} and cps_{s3} are the activities recorded during the silicone periods $s1$, $s2$ and $s3$, which followed the Ringer perfusion periods $r1$, $r2$ and $r3$. It is further assumed that the "trapped volume" and the surface film (O_R) had attained the specific activity of the active Ringer solution instantaneously at the first active Ringer perfusion period ($r1$). The different time scales have been

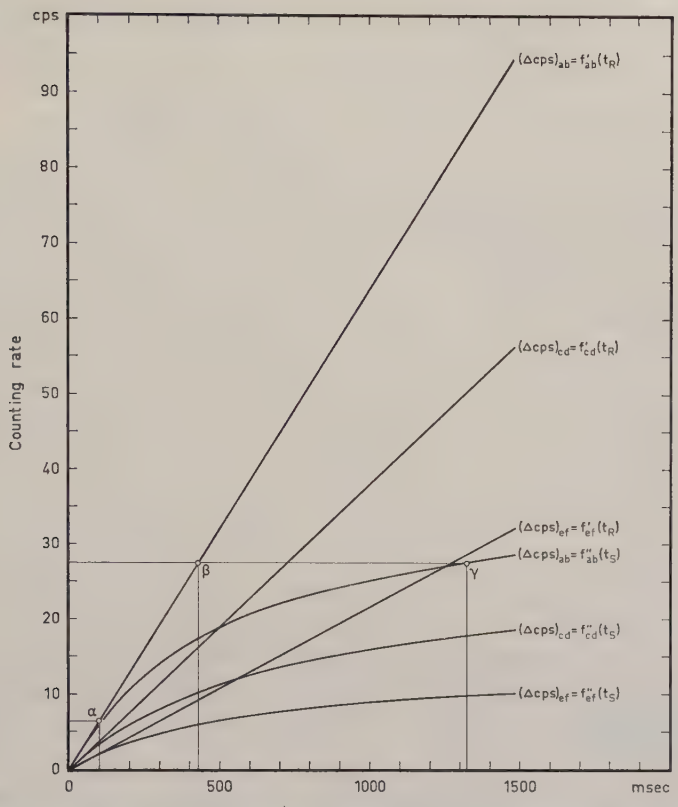

FIG. 37. The increase in activity in the tissue of the preparation during different stages of the influx experiment of Fig. 32 (p. 11). Values for both Ringer and silicone oil perfusion are given. The increase of activity during active Ringer perfusion does not follow the same time course as during silicone perfusion. The figure can be used as a *nomogram* for the calculation of $t_{r_{eqv}}$ in experiments where t_r and t_s have different values. $t_{r_{eqv}}$ is the intercept (β) of the estimated counting rate (γ) at t_s with the increase in counting rate with active Ringer solution alone.

illustrated schematically as in Fig. 34 (p. II), i.e. for curve IV during Ringer and silicone oil phases during alternate perfusion (top time diagram under abscissa) and for curve III under hypothetical continuous Ringer perfusion ($t_r + t_{r_{eqv}}$). The upper part of the left ordinate represents the Na^{24} activity in the tissue. The increases of activity are assumed to be identical in each of three Ringer-silicone oil cycles; the increase during the Ringer period ($t_r = 100.4$ msec) is 6.4 cps and in the silicone period ($t_s = 1320$ msec) 27.4 cps as calculated in Fig. 34

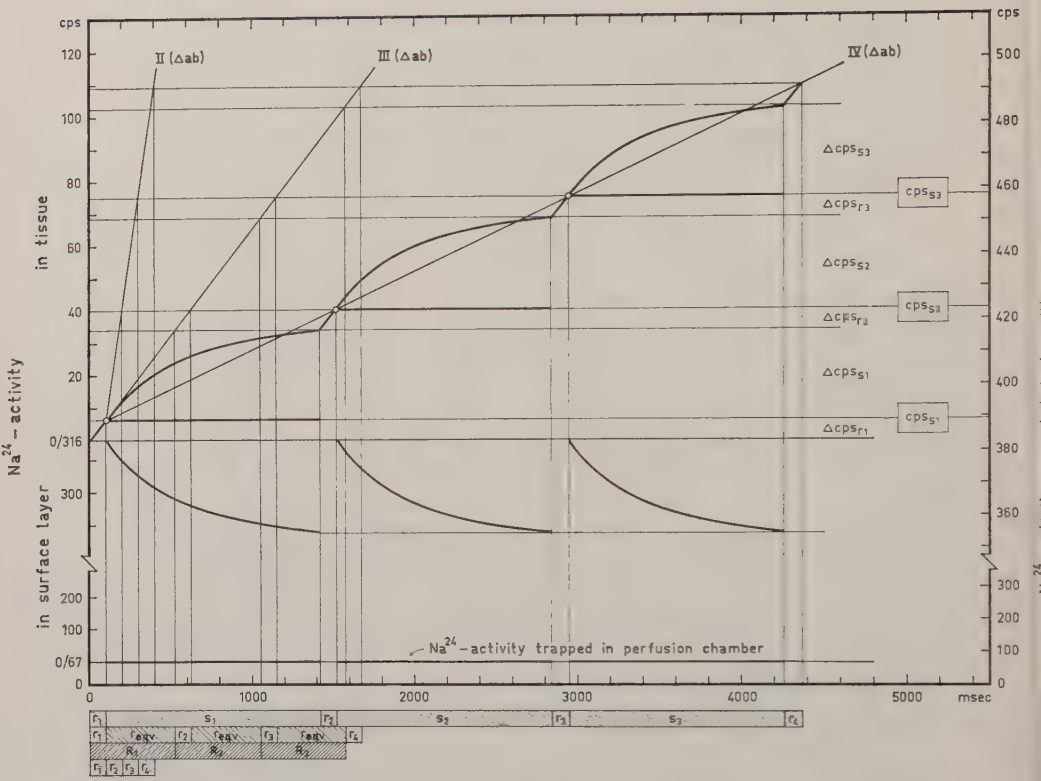

FIG. 38. Reconstruction of a small part of the interval *ab* of Fig. 32 (p. II). The right ordinate gives the total activity recorded after subtraction of the background (background = 186 cps corresponding to 1.17 mg active Ringer solution). *Note.* Both the left and right ordinate scales are interrupted. For further details see text.

(p. II) and Fig. 37 (p. 79). The activity recorded during each silicone period is arbitrarily always placed at the beginning of the period ($t_s = 0$, open circles), the point of time at which the activity becomes "locked in" by the silicone (passage of Ringer–silicone phase boundary). There is assumed to be no loss of activity from the chamber during the silicone oil perfusion period.

If V_o and the "trapped volume" are constant (or vary purely randomly from cycle to cycle) the activity in the tissue can be determined with the greatest certainty at $t_s = 0$, when under the above conditions it is correct to write

$$\text{cps}_{\text{tissue}} = \text{cps}_s - (\text{cps}_{O_R} + \text{cps}_{\text{trapped activity}} + \text{cps}_{\text{background}});$$

where cps_s = total activity recorded during a silicone oil period and cps_{O_R} = total activity of surface film O_R at $t_s = 0$, and

$$(cps_{O_R} + cps_{\text{trapped activity}} + cps_{\text{background}}) = \text{constant}.$$

During silicone oil perfusion there will be some redistribution of radioactivity between the surface film (O_R) and the tissue. The tissue increase (Δcps in Fig. 38) cannot be determined before the next analysis period during the succeeding silicone period. The same amount of tracer (Δcps_s) must also leave O_R. During the active Ringer perfusion there will be a net tracer movement (Δcps_r) into the tissue compartment, and after each such period the total activity in the surface layer (O_R at $t_s = 0$) will be 316 cps.

The tissue activity (cps_{tissue}) *during the silicone–Ringer cycles can be described in the average case by the function represented by the thick line between the open circles in the figure. The difference in activity recorded between two analysis points depends on the particular type of distribution function operating during the intervening period.* This also imposes limits on the time intervals during an influx experiment for which measured activity differences are representative.

In the influx experiments the entire activity measured during a silicone oil perfusion period is assigned to the end of the previous Ringer period (r)*; the maximal conceivable shift of the point would be by a time* ($t_r + t_s$) *to the left, i.e. one whole silicone-Ringer cycle earlier.*

With the use of a *c-a-t* it is implicit in applying the interpolation procedure that the distribution functions in all the individual silicone–Ringer cycles are the same (chapter VI, e).[1]

The assumptions regarding the constancy of the volume of the surface layer O_R requires further comment. *Since the activity during silicone oil perfusion is virtually constant* (see summary of section B), *the volume* (V_o) *of* O_R *will be determined when the Ringer–silicone phase boundary passes the tissue preparation. If the volume of* O_R *is correlated to the phase of the mechanical activity the latter must also be correlated*

[1] The whole of the increase of activity in the preparation (≈ 34 cps) measured as the difference between two successive measurements occurs before the time to which the increase is attributed. About 23 % of the influx occurs linearly during t_r [$t_r = 0.076$ ($t_r + t_s$)] and the remaining 77 % exponentially from O_R during the silicone oil phase (t_s). Thus the 50 % influx point occurs earlier than the mid-point of the silicone-Ringer cycle preceding the time point to which the subsequent measurement is assigned.

with the passage of the interface (i.e. the time point to which radioactive measurements have been assigned in all influx experiments).

The DC and AC components during an influx experiment

In an influx experiment with alternate Ringer–silicone oil perfusion (DMM) of a sinus venosus preparation mounted in a chamber, the radioactivity recorded increases with respect to both the times of perfusion of the Ringer and silicone oil. If all fluctuations are neglected an exponential mean curve is obtained ("DC component", see chapter VI, e). The influx kinetics during the individual Ringer and silicone oil periods are different; this presumably depends on different boundary conditions. During the Ringer period the preparation is surrounded by a solution of constant specific activity (infinitely large source). In the silicone oil phase the tracer can only distribute between the tissue and a surface layer of limited volume whose specific activity must decrease (providing the tracer diffuses down its concentration gradient).

The time course of the DC component depends on 1) the electro-mechanical state of the tissue and 2) the perfusion programme, i.e. the relationship between the lengths of the Ringer (t_r) and silicone oil (t_s) periods i.e. t_r/t_s. As can be seen in Fig. 24 (p. 52) the DC curve was steeper in a spontaneously beating than in an electro-mechanically inactive preparation. Assuming t_r/t_s remains constant during the whole experiment the DC component with a non-beating preparation should represent the result of a "passive" redistribution of tracer in the tissue under the perfusion conditions operating ("passive distribution base line"). With increase of t_r/t_s the slope of this "passive" DC line should be steeper; the steepest curve obtained was that during continuous Ringer perfusion (cf. curve III in Fig. 32, p. II, and curve II in Fig. 25, p. I).

Superimposed on the DC line is an "AC component" (see chapter VI, e). *Fluctuations around the mean trend line* (*= base line*) *consist to a great extent of "random noise"* (see chapter VI, c), i.e. the resultant of variations in the analysis system, the perfusion system ("trapped activity"), background activity, random variations in the value of the extra-cardiac surface layer (O_R) and the statistical counting error. *It was calculated with the c-a-t method that the AC component also exhibited systematic fluctuations of radioactivity correlated with the electro-mechanical activity cycles of the spontaneously beating preparation.*

Systematic fluctuations in the activity of the spontaneously beating preparation might arise from events not directly connected with transmembrane ion movements, i.e. 1) there may be a correlation between the volume of the external film (O_R) during silicone perfusion and the mechanical activity of the preparation, and 2) *the distribution and movements of tracer with the tissue may depend on both mechanical* (e.g. geometrical changes of extracellular matrix) *and electrical events* (e.g. causing alterations in the physico-chemical properties of the extracellular space).

The time relations between the measured radioactivity pattern and the electro-mechanical activity for a "mean cycle"

The radioactivity measured during the silicone period (DMM) is arbitrarily assigned to a time point at the very beginning of that silicone oil period, i.e. at the termination of the period whose influx is being determined. The start of this period was $(t_r + t_s)$ seconds earlier.

If the tissue activity pattern depends on some correlation between O_R and the phase of mechanical activity a time correction for the cycles of the latter is not necessary because the radioactivity measurements are made during a period when the total activity does not change (t_s) and assigned to the time at which the Ringer–silicone interface passes (cf. p. 81).

If the fluctuations estimated by the c-a-t are due solely to variations in the volume of O_R then under all circumstances the patterns for all isotopes studied by the DMM will have the same general relationship to the electro-mechanical events independently of the relationship between t_r and t_s.

If the distribution of the tracer in the tissue is affected by the electro-mechanical activity then different isotopes would quite likely not behave in the same way, and the activity pattern would probably be correlated with the t_r/t_s ratio. In this case a correction for the time relation between the mean activity cycle and the electro-mechanical events might be necessary (though difficult to calculate). *It seems probable that the mean cycles for Br^{82} and Na^{24} (Figs. 26, p. 56; 47, p. 117 and 48, p. 118) should be placed a little earlier in relation to the electro-mechanical cycle* (cf. general discussion, p. 120).

Summary: The time course of the radioactivity recorded in an influx experiment (DMM) on a spontaneously beating sinus venosus

has two components, a mean exponential (DC) curve on which are superimposed fluctuations (AC component). The form of the DC component depends on the electro-mechanical activity of the preparation and the perfusion conditions (t_r/t_s). The AC component consists both of "random fluctuation noise" and systematic fluctuations correlated with the electro-mechanical activity of the spontaneously beating tissue. Systematic fluctuations in the radioactivity around the base line might arise from processes not directly connected with transmembrane ion movements. There may be a correlation between the volume of O_R and the mechanical activity of the preparation, or some relationship between the electro-mechanical activity and the distribution of tracer in the preparation. If the activity pattern calculated by the *c-a-t* method resulted solely from variations in O_R, the activity patterns for the different isotopes studied with the DMM should have essentially the same form and relation to the electro-mechanical events. If there is a correlation between the electro-mechanical activity and the distribution of tracer in the tissue, it is likely that with different isotopes both the pattern of radioactivity and its relationship to the electro-mechanical events would differ. Further, in the latter case the relationship between the electro-mechanical events and the radioactive pattern would probably depend on the perfusion conditions (the value of the t_r/t_s ratio).

D. The equilibration volumes and uptake rates during an influx experiment

A review and analysis of ion exchange kinetics between two different phases in a multi-compartment system has been recently published by Haas (1962). By way of introduction to the experiments which were carried out with the perfusion and soaking methods described in chapter V, a short review of certain relevant literature concerning the distribution of electrolytes and non-electrolytes between bathing solutions and tissue preparations will be given. Because of the type of preparation and perfusion techniques used, the results vary within rather wide limits.

Carey and Conway (1954) showed that a rapid sodium efflux from the frog sartorius probably came from the extracellular space. Schreiber (1956) described both influx and efflux experiments on frog ventricle, and concluded that the K^{42} efflux had two components,

a rapidly escaping fraction with $T\frac{1}{2}=22$ min and a slower fraction with $T\frac{1}{2}=80$ min; similarly for the influx there were also two velocities involved, a rapid component with $T\frac{1}{2}=15$ min and a slower one with $T\frac{1}{2}=68$ min. The Na^{24} efflux was also dual in nature, with a rapid component (comprising 94 % of the total Na^{24}) with $T\frac{1}{2}=4$–4.5 min (interpreted as representing the extracellular Na^{24}), and a slower fraction with $T\frac{1}{2}=170$ min. Johnson (1957) found that the Na^{22} influx into the frog ventricle reached a plateau within 10 minutes. Harris (1957) claimed that equilibration of the extracellular space in the toe muscle (weight about 3 mg) from the frog took place within 1 min. Hutter (1961) found that the efflux of K^{42} from the extracellular space in the sinus venosus of the frog was virtually complete within three minutes; with perfusion of frog ventricle Gray, Lamb and McGuigan (1964) found rapid clearing of the extracellular space. Haas and Glitsch (1962) studied the potassium flux in frog auricle (thickness about 0.3 mm), and both the influx and efflux were of a double exponential type; the $T\frac{1}{2}$ value for inulin efflux was 1.7 min. Haas, Glitsch and Trautwein (1963) studied Na^{24} efflux from frog auricle and claimed that there were three components; the first rapid fraction was interpreted as being due to expulsion from the extracellular space (it was dependent on electro-mechanical activity). Langer and Brady (1963) found that in the mammalian ventricle the influx of calcium reached a steady value within 20–25 min. Niedergerke (1963) studied Ca^{45} influx into frog ventricle; in a normal sodium environment Ca^{45} reached a steady level within 5 minutes, while the sulphate, inulin and sodium spaces were about the same size, the calcium space was larger. The Ca^{45} efflux was correlated with the stimulation frequency.

Equilibrial volumes and times for soaking experiments

Fig. 39 illustrates an experiment where a spontaneously beating atrium and sinus venosus were bathed for different periods in active Ringer solution which contained Na^{24}, Br^{82} and raffinose (solution III, c). The solution was vibration-stirred and the method was as described in chapter V, A, b. The experiment was one of two which were made, both yielding the same results. The equilibration volumes were calculated, assuming that they contained the same concentration of test solute as in the active Ringer solution,[1] and the data are

[1] No correction was made for the Donnan distribution of electrolytes be-

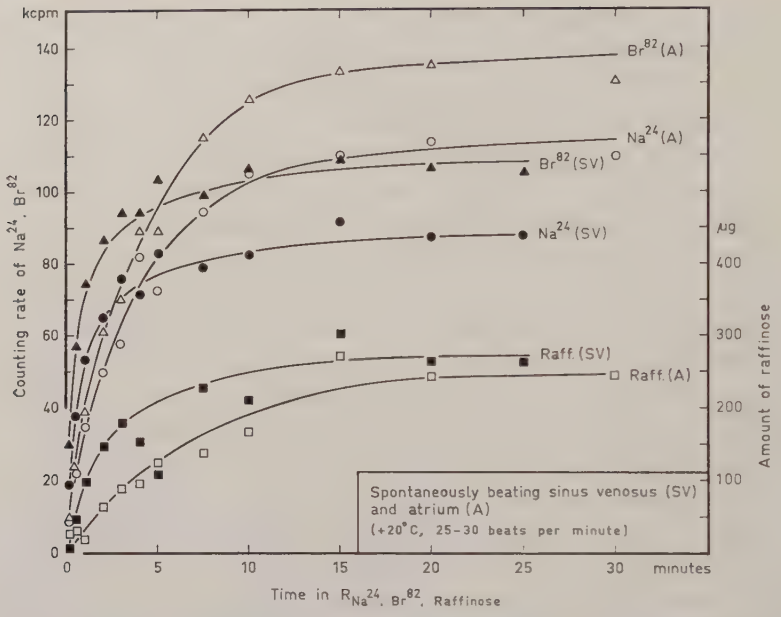

FIG. 39. Influx experiment for Na^{24}, Br^{82} and raffinose on a spontaneously beating sinus venosus (SV) and atrium (A) by the procedure described in chapter V, A, b. Wet weights: sinus venosus 14.0 mg, atrium 20.8 mg. Analysis accuracy for Na^{24} and Br^{82} better than $\pm 0.5\%$. Activities of solutions: $[Na^{24}] = 7093$ cpm/mg, $[Br^{82}] = 7921$ cpm/mg; the raffinose concentration was 20 µg/mg. (ESA values: 85 kcpm Na^{24}/µmole Na, 281 kcpm Br^{82}/µmole Br.) External surface layer (O_R) calculated as 8.5% of the wet weight: $(O_R)_{SV} = 1.19$ mg equivalent to 8.4 kcpm Na^{24}, 9.4 kcpm Br^{82} and 24µg raffinose. $(O_R)_A = 1.77$ mg equivalent to 12.6 kcpm Na^{24}, 14.0 kcpm Br^{82} and 35 µg raffinose. The equilibration levels in the figure are: Sinus venosus (SV): Na^{24} 82.5 kcpm, Br^{82} 102.5 kcpm and raffinose 260 µg. Atrium (A): Na^{24} 107.0 kcpm, Br^{82} 130.0 kcpm and raffinose 235 µg. (1 kcpm = 10^3 cpm.)

given in the figure text.[1] It can be seen in the figure that Na^{24}, Br^{82} and raffinose equilibrated relatively rapidly with a space which was considerably greater than that of the extra-cardiac surface film (cf. Fig. 28, p. 65). In general it can be said that the equilibration level was reached more rapidly for sinus venosus than for atrium. The

tween Ringer solution and the extracellular space of the preparation; the theoretical Donnan factor for human plasma and protein-free ultrafiltrates has been calculated and found to be approximately 0.96 for univalent ions (for reference, see BERGSTRÖM, 1962). Cf. also DANIELSON (1964).

[1] The "backward projection" technique for graphical resolution of exponential processes in the determination of the rate constants was not used here because of the rather "coarse" technique used, see LIEW (1962).

longer equilibration times for atrium depend probably on the larger equilibration volume. It is also clear that the distribution of raffinose in both cases was slower than that of Na^{24} and Br^{82}. This is not unexpected in view of the different diffusion coefficients for the substances.[1]

The raffinose-Ringer solution was hypertonic (raffinose concentration = 34 mM). Bozler (1961 *b*), with frog sartorius, showed that a sucrose-Ringer solution (80 mM sucrose) produced a reduction in weight of about 14 % after a soaking time of 2 hours. It was shown also that sucrose entered into the extracellular space and probably also intracellularly (Bozler, 1959, 1961 *a*). The weight loss recorded therefore is presumably the resultant of a rather complex system of forces. The osmotic effect of the raffinose-Ringer solution (34 mM raffinose) was therefore studied in a soaking experiment with the determinations of the changes in wet weight after different lengths of soaking time. Inverted, spontaneously beating sinus venosus and atrium were mounted in the same way as shown in Fig. 22; a toe muscle from the toad was also used. After soaking for 30 minutes at room temperature the wet weight reductions were for sinus venosus 7 %, atrium 5 % and toe muscle 5 % (of the original weight in normal Ringer solution—solution I); after 2 hours' soaking the values were 15 %, 8 % and 10 % respectively.

The equilibration levels shown in Fig. 39 (see figure text) include an extra-cardiac surface layer, O_R, which was calculated as equivalent to 1.19 mg of solution for the sinus venosus preparation and 1.77 mg for the atrium (8.5 % of the wet weight). After subtraction of the quantity of "activity" in O_R the equilibration volumes for the preparations were calculated (cf. footnote p. 85) as sinus venosus: raffinose = 11.8 mg, Br^{82} = 11.8 mg; atrium: raffinose = 10.0 mg, Br^{82} = 14.6 mg.

Danielson (1964) measured the bromide space after 30 hours' equilibration *in vivo* (*Bufo bufo*), and using his values (calculated without a correction for the Donnan distribution), the Br^- space in the sinus venosus used above should correspond to 9.3 mg $\pm$ 1.1 mg, and in the atrium 11.3 mg $\pm$ 1.0 mg (the Br^- and I^- spaces were essentially the same as that of Cl^- in sinus venosus, auricle, ventricle

[1] D_{NaCl} (concentration = 100 mM) = 1.48×10^{-5} cm^2 sec^{-1} and D_{NaBr} (100 mM) = 1.51×10^{-5} cm^2 sec^{-1}, at 25°C (Robinson and Stokes, 1955), and $D_{raffinose} = 0.42 \times 10^{-5}$ cm^2 sec^{-1} at +18°C (Conway, 1952).

and skeletal muscle). Assuming that Br^{82} is mainly extracellular the equilibration volumes for Br^{82} and raffinose for the sinus venosus are in good agreement, though perhaps somewhat greater than DANIELSON's values. As regards the atrium there is good agreement between the equilibration volume for raffinose and the extracellular space, but the equilibration volume for Br^{82} is higher than given by DANIELSON. A calculation of the equilibration volume for Na^{24} in the experiment of Fig. 39 gave for the sinus venosus 10.4 mg, and for atrium 13.3 mg; the values calculated from DANIELSON's (1964) data were 8.8 ± 1.3 and 11.2 ± 0.9 respectively. Although the technique used was rather coarse, the results are in general in good agreement with those reported in the literature, though two potential sources of error must be noted: The volume of O_R was not determined for each individual preparation, and further, in determining the wet weight any Ringer solution which was trapped between the epicardial surface of the preparation and the ECG catheter was not taken into account (cf. Fig. 15, p. 34).

To summarize it may be said that the equilibration volumes for Na^{24}, Br^{82} and raffinose in sinus venosus were in good agreement with the extracellular anion space estimated by DANIELSON. In the atrium the agreement was good for raffinose but there were small differences for Br^{82} and Na^{24}. The rate of equilibration of the extracellular space in sinus venosus was in good agreement with the value given by Hutter (1961). To what extent the intracellular space is involved in the experiment of Fig. 39 is difficult to assess. For sinus venosus and atrium the intracellular quantitites of Na and Br are, according to DANIELSON (1964) very small, and with the method used for the experiments of Fig. 39 would therefore not be measurable.

Equilibrial volumes and times for perfusion experiments (DMM)

The volumes and rates of equilibration obtained with the perfusion technique at the temperature which was used for the influx experiments (DMM) require some comment. In, for example, a Na^{24} (Fig. 32, p. II) influx experiment, the true influx curve during continual active Ringer perfusion was calculated (cf. Fig. 32, curve III).

For the *Br^{82} influx experiment* (Fig. 25, p. I) $t_{r_{eqv}}$ was calculated from Fig. 37 (p. 79) with the assumption that the $D_{NaBr} \approx D_{NaCl}$ (cf. footnote on p. 87), and the correction of the time scale

was made by using the value of $t_{r_{eqv}}$ so obtained (time scale for curve II in Fig. 25 can be derived from the equation given in the figure). The equation for describing curve II in Fig. 25 was obtained by the same procedure as used for determining that of the mean trend line, curve I, in the same figure (see chapter VI, e); the equilibration level corresponded to 7850 cps obtained at $(t_r + t_{r_{eqv}}) < 200$–$250$ seconds (+5°C); the equilibration volume after subtraction of the activities of the trapped volume and the extra-cardiac surface film (O_R) was calculated as 12.3 mg. The value calculated from DANIELSON's (1964) data was 10.7 ± 1.3 mg.

As is evident from Fig. 32, curve III, the *Na^{24} influx experiment* did not reach an equilibrial value. In order to assess the magnitude of the equilibrial level, the equation for curve III, describing the relation between increase in activity in the preparation and perfusion time in active Ringer solution, was calculated in a similar way as curve II of the Br^{82} experiment of Fig. 25 (p. I). The equation could then be used to calculate the *approximate* values for the equilibrial level and time; the equilibrial level corresponded to 3550 cps, and was reached at $(t_r + t_{r_{eqv}}) \approx 200$–$250$ sec (+7.5°C). After subtraction for the activity of the trapped volume and O_R it was calculated as 19.8 mg. The value calculated from DANIELSON's (1964) data was 14.8 ± 2.2 mg.

The calculated equilibrial volumes for Br^{82} and Na^{24} (DMM) thus are in essential agreement with the results of the soaking experiments (Fig. 39, p. 86). The somewhat higher values for the equilibrial volumes for Br^{82} and Na^{24} can perhaps be explained by the uncertainty in the calculated volume of the extra-cardiac surface film, and the fact that with determination of the wet weight of the preparation any volume of Ringer solution trapped between the epicardial surface of the preparation and the ECG catheter was neglected (cf. Fig. 15, p. 34).

In the influx experiment made with solution I or II (DMM), no great variation between either the volumes of the intra- and extracellular spaces or the ion concentrations should be expected. JOHNSON (1957) showed that the sucrose space was constant for about 2 hours when a frog ventricle was perfused with normal Ringer solution; intracellular Na concentration was also apparently unaltered.[1]

[1] CAREY and CONWAY (1954) showed, however, that the Na and Cl content in isolated sartoris muscles increased with time during equilibration in Ringer

Summary: In the influx experiments made by the DMM it is probable that with a perfusion temperature of between +5°C and +7.5°C the uptake of tracer is primarily in the extracellular space (equilibrial time for sinus venosus about 200–250 sec during continual Ringer perfusion). It is probable that the uptake rate during perfusion (DMM) was somewhat higher than is the case with more conventional techniques (soaking experiments, see Fig. 39, p. 86), where equilibration of the extracellular space in sinus venosus at about +20°C took place within 3–6 min for Na^{24} and Br^{82}. It appears therefore that a somewhat more rapid uptake of the tracer occurs with the perfusion method (DMM) than with conventional soaking.

E. Studies of the distribution of K^+ ions between the perfusion solution and the outermost cells of the tissue preparation

A method has been previously described for the determination of the size order of the time lag which can be expected with influx measurements (Danielson, Öbrink and Sjöstrand, 1962).

A high outer concentration of K^+ is known to depolarize excitable membranes (for references cf. Higman, Podleski and Bartels, 1963), including heart tissue (Weidmann, 1956 *a*, *b*). With a K^+ rich isotonic Ringer solution Kotowski, Antoni, Vahlenkamp and Fleckenstein (1961) found that the minimal K^+ concentration for depolarization of the frog sinus venosus was about 22 mM K^+.

The time interval between contact of the preparation with a Ringer solution containing 44 mM K^+ (solution IV) and depolarization (estimated from the ECG) should give some information about the proximity of the outermost cells to the perfusion solution (cf. chapter II, pp. 13, 15). The main part of this time difference can probably be attributed to the time elapsing between contact of the preparation with the potassium Ringer solution and the depolarization of the outermost cells, which thus acted as a trigger focus for the rest of the tissue. The outermost cells are, however, those of greatest interest, since their superficial position presumably means that they will be very much involved in any intracellular influx which occurs.

The sinus venosus (or atrium) was mounted as described in chapter

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solution. Haas, Glitsch and Trautwein (1963), in a perfused toad atrium, found that during the first two hours the total Na increased, while the K decreased.

TABLE 4. *K^+-depolarization time lag at $+5°C$.*

Exper.[a] No	Preparation	K^+ time lag in msec	Number of determinations	Range
640319 I	sinus venosus	270	6	230–330
640319 II	sinus venosus	170	6	130–220
640319 V	sinus venosus	140	19	85–180
640319 III	atrium	370	3	340–480

[a] Each experiment represents measurements on one preparation.

II, b, and perfused in the same way as with the DMM (see chapter V, A, a). The preparation was perfused with inactive Ringer solution and then with silicone oil (the ECG was recorded continuously). The silicone oil perfusion was interrupted by a perfusion period with K^+ Ringer (solution IV, 44 mM K^+). The time of first contact of the K^+ Ringer solution with the tissues of the preparation could be determined with the technique described in chapter IV, D, a, 2. The mean lag time found in this way was for sinus venosus perfused at $+5°C \approx 200$ msec (see Table 4).

The time lag measured as described above presumably corresponded to the time required for the potassium ions to reach the cellular membranes together with the time required for their depolarization (depolarization should occur when the extracellular K^+ concentration is about 22 mM).[1] Even if the depolarization time of the membrane varies depending on the phase of the cardiac cycle when the high potassium concentration reaches the cell membrane, the distribution time between the perfusion solution and the cellular membrane will be the same in all cases. Thus the real time lag will never exceed the observed values. DANIELSON, ÖBRINK and SJÖSTRAND (1962) found that the time lag for sinus venosus was 100 msec, and for atrium 400 msec, using a perfusion technique, most nearly resembling that used

[1] In studies of the effect of potassium on the depolarization of the electroplax of *Electrophorus electricus*, HIGMAN, PODLESKI and BARTELS (1963) found that the membrane potential appeared to be most sensitive to changes in potassium concentration.

in the experiment where the preparation was bathed in a soaking solution (cf. Fig. 39, p. 86). The potassium concentration used in this experiment was 125 mM K^+, which was considered unnecessarily high.

F. Electro-mechanical dissociation, the effect of the mechanical activity on the influx (DMM) patterns for Na^{24} and Br^{82}

It seems quite possible that the mechanical activity can affect both the influx and efflux patterns by causing variations in the surface volume relationship during the contraction phase (see chapter I). This in its turn might give rise to mechanical artifacts by 1) extracellular "squeezing" and 2) "squeezing" of the extra-cardiac surface layer (cf. general discussion). Electro-mechanical dissociation would give possibilities for studying the effect of the mechanical activity on the influx patterns for Na^{24} and Br^{82}.

Electro-mechanical dissociation has been described, *inter alia*, by Antoni, Engstfeldt and Fleckenstein (1960) for frog ventricle muscle by reduction of the Ca^{++} content of the soaking solution. Winegrad (1961) discusses the importance of the Ca^{++} ions in an electro-mechanical coupling.

According to Weidmann (1955) reduction in Ca^{++} concentration does not affect the resting potential; different Ca^{++} concentrations varying from $\frac{1}{4}$ to 4 times the normal did not affect slow depolarization in a pace-maker region (in this concentration range a change of frequency is only produced by a change in the threshold potential).

A low calcium environment does not only affect the electro-mechanical coupling. Weidmann (1955), with a "voltage clamp" technique, showed that the Na permeability during depolarization was reduced if the calcium concentration was lowered.

Experiments were made on isolated, spontaneously beating toad auricles mounted as described for the "sampling method" (chapter V, B, a). The ECG was recorded in the usual way, and the mechanical activity (mechanogram) as described in chapter IV, A, b. In some cases the action potential was also recorded with a suction electrode. The results of such an experiment are shown in Fig. 40, where all the action potentials were measured with an electrode which was neither moved nor released from the tissue during this period. It can be seen that the amplitude of the action potential decreases with "sucking

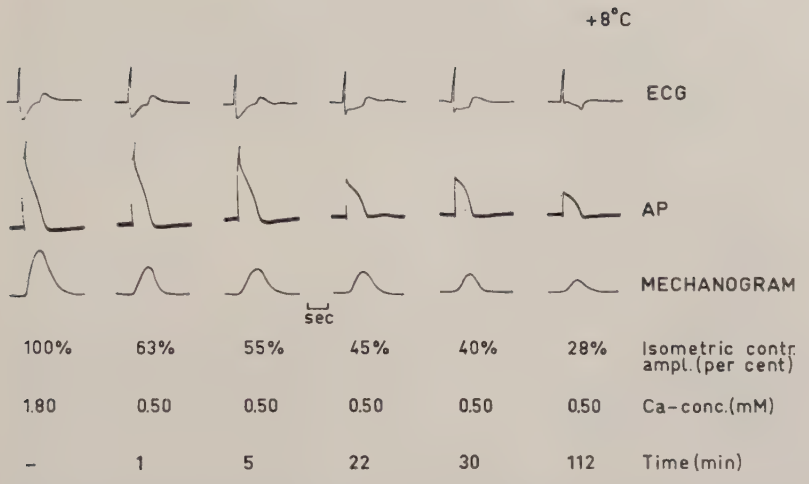

Fig. 40. Recording of ECG, action potential (suction electrode) and mechanogram (isometric) on a spontaneously beating atrium. The isometric amplitude after bathing for different periods in a solution containing 0.5 mM Ca^{++} is given.

time", in accordance with the findings of Schütz (1931). A new "sucking" after about 115 min from the beginning of the experiment gave an action potential, which had about the same amplitude as that of the first in Fig. 40. The mean amplitude of the isometric contractions with perfusion of Ringer solution containing 0.5 mM Ca^{++} (solution I with reduction of the Ca^{++} concentration) was for six atrial preparations 25.4 % (range 18.5–27.5) of that with 1.8 mM Ca^{++}. In all cases the electro-mechanical dissociation was reversible. In one case perfusion was made with a Ringer solution of 0.4 mM Ca^{++}, which induced a reversible reduction of the contraction amplitude to 15.5 % of the original value. In two preparations perfused with 0.25 mM Ca^{++} the mean amplitude was reduced to 9.9 %, but subsequent perfusion with 1.8 mM Ca^{++} did not fully restore the contraction amplitude. All experiments were made at a temperature of +8°C.

Reducing the calcium concentration from 1.8 to 0.5 mM Ca^{++} usually lengthened the duration of the action potential by 10 %. The time relations of the contraction cycle (start and maximum contraction) to the ECG and action potential were the same with normal and low calcium concentrations.

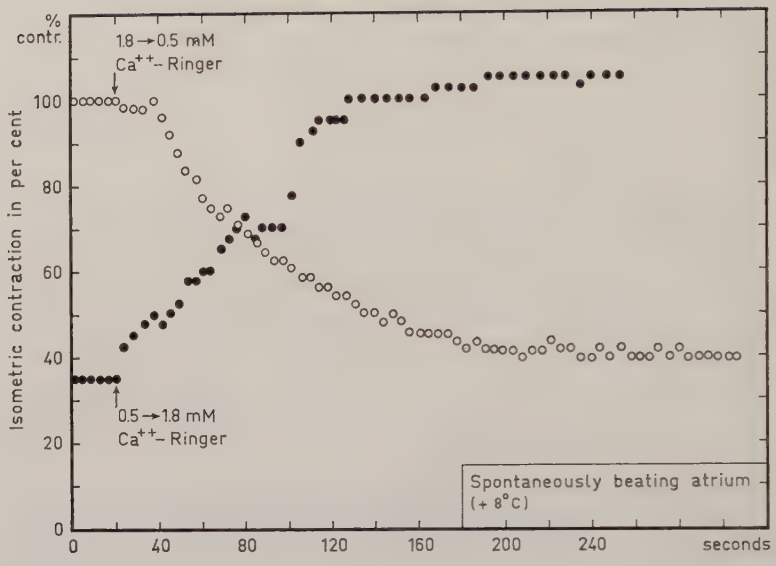

FIG. 41. Isometric contraction amplitude (in per cent of that obtained in 1.8 mM Ca^{++}) of a spontaneously beating atrium in a Ringer solution containing 0.5 mM Ca^{++} (open circles). When the concentration was restored to 1.8 mM Ca^{++} the contraction amplitude returned to its original level (solid circles). The recovery curve has been drawn on the same time scale as the first curve though in fact it was subsequent to it.

Fig. 41 shows the electro-mechanical dissociation for a spontaneously beating atrium during perfusion with a Ringer solution containing 0.5 mM Ca^{++}; the amplitude of contraction at 1.8 mM Ca^{++} has been taken as the 100 % level. Perfusion was begun with 1.8 mM Ca^{++}, and then suddenly switched to 0.5 mM Ca^{++}; later, when the contraction amplitude value had been steady for some time, the perfusion was switched back to 1.8 mM Ca^{++}. The results allow an approximate estimate of the equilibration rate for calcium in the preparation; the value was similar to that found by NIEDERGERKE (1959).

Preliminary studies indicate that the electro-mechanical dissociation induced by reduction of the Ca^{++} concentration is similar in sinus venosus.

No *influx* experiments have as yet been made on the preparation with reduced amplitude of contraction, and therefore the effect of the

mechanical activity on the extra-cardiac surface film cannot be assessed *directly*. Other experiments which give *indirect* information were made with a contraction amplitude of only 25 % of the normal; these experiments will be described later (see efflux experiments, Fig. 46 *a–c*, pp. 107–109).

EFFLUX EXPERIMENTS

VIII. *The efflux of tracer from the sinus venosus and atrium under different perfusion conditions*

Some of the literature relating to ion fluxes during single action cycles in heart muscle was reviewed in the introduction. It has been claimed that K^{42} efflux shows cyclical variations in relation to the heart cycle (Wilde and O'Brien, 1953; Danielson, Öbrink and Sjöstrand, 1961, 1962; Lorber *et al.*, 1962); similar results have also been obtained for Na^{24} efflux (Danielson, Öbrink and Sjöstrand, 1961, 1962). The common working hypothesis in these experiments has been that the radioactive pattern in a perfusate reflects the ion flux between the intra- and extracellular spaces in the tissue, even if both damping phenomena and time lags make the results uncertain and difficult to interpret (the type of preparation and perfusion technique used often make it difficult to compare results). In all the works discussed there is the possibility that the efflux patterns resulted from mechanical activity. A mechanical "squeezing" effect has been considered very unlikely on *indirect* grounds, but *no direct* proof that the ion flux pattern is independent of mechanical activity has been forthcoming.

To obtain optimal conditions in an efflux experiment with the "wash-out" technique, it is a requirement that the movement of tracer across the boundary between the preparation and the perfusion solution is not the rate-limiting step. *Perfusion of the preparation must therefore be arranged so that the unstirred layer which surrounds it is as small as possible* (in those cases where the preparation has a mechanical activity the unstirred layer should in addition not vary significantly in relation to the mechanical activity phase). *A turbulent flow with a high and constant linear mean velocity should therefore be expected to give the best distribution conditions between the preparation and the perfusion solution.*

In order to study the importance of the perfusion conditions for

the distribution kinetics of the tracer between the preparation and the perfusion solution, the relationship between perfusion velocity and the efflux pattern has been studied.

The movement of tracer from the non-beating preparation to the perfusion solution at different perfusion velocities

Fig. 42 shows the results obtained on a non-beating sinus venosus (the preparation did not beat at a perfusion temperature of +5°C); the method used was that described in chapter V, B, a. The sinus venosus was loaded in radioactive Ringer solution (Na^{24}, Br^{82}) for one hour at room temperature with continuous vibration stirring (at this temperature it beat spontaneously). Two efflux experiments were made on the same preparation after loading in the radioactive Ringer solution (see chapter V, B, a). The first experiment (curve I in the figure) was made with a low perfusion velocity (about 0.8 g perfusion solution per sec corresponding to $\bar{v} \approx 35$ cm/sec). After a second loading in the radioactive Ringer solution (1 hour) the second efflux experiment (curve II) was made at a high perfusion velocity (about 6 g perfusion solution per sec corresponding to $\bar{v} \approx 265$ cm/sec). Fig. 42 shows the total amounts and concentrations of Na^{24} and Br^{82} in the perfusate (the analysis accuracy for Na^{24} and Br^{82} were the same), and also the amount of perfusate in each individual sample. The ratio between the total Br^{82} and Na^{24} in each sample is given in the figure. *In the discussion which follows the assumption is made that the specific activity of the preparation was the same at the beginning of the two efflux experiments.*

As expected, the concentrations of Na^{24} and Br^{82} in the perfusate were higher with a low than a high perfusion velocity. The middle section of the figure shows that the rate of efflux of the tracers was greater during a perfusion at the higher velocity (amount = concentration times sample volume). At both perfusion velocities the ratios between the total amounts of Br^{82} and Na^{24} in each sample were relatively constant (this shows that the relative depletion of the two isotopes was the same during the period of the experiment); at the higher velocity, however, there was a somewhat higher Br^{82}/Na^{24} ratio in the perfusate.

Fig. 43 shows an efflux experiment made on a non-beating sinus venosus which was loaded for one hour at room temperature with

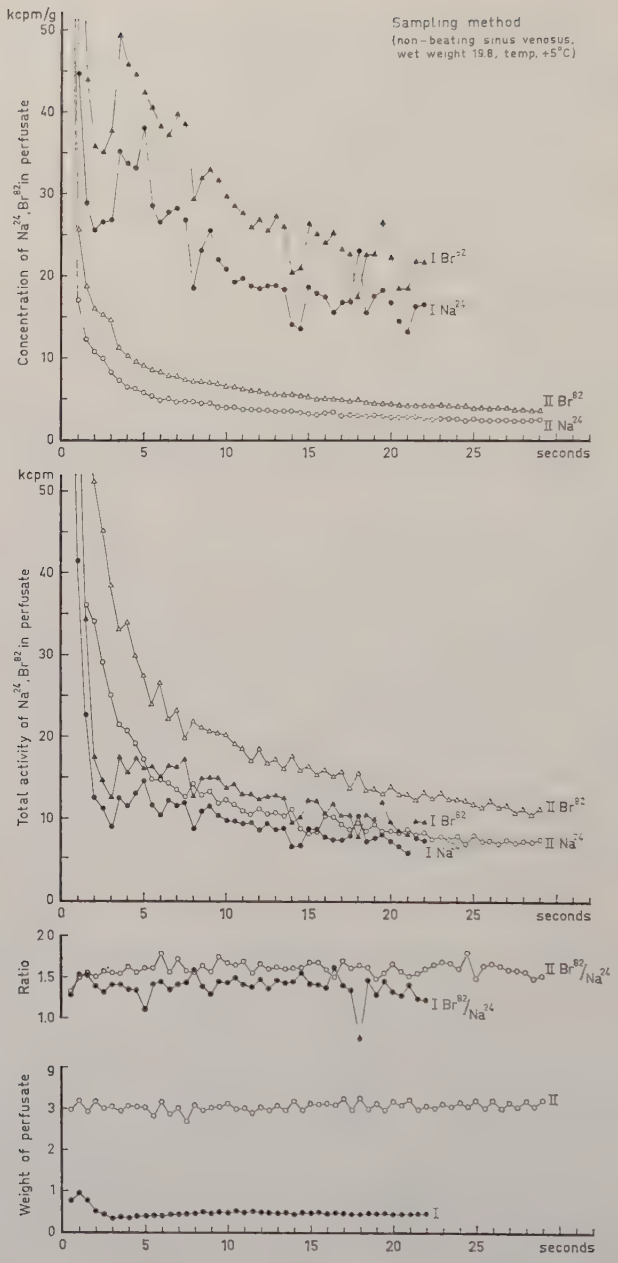

FIG. 42. Two efflux experiments for Na^{24} and Br^{82} on a non-beating sinus venosus by the method described in chapter V, B, a. The accuracy of the analysis was for Na^{24} better than $\pm 2.5\%$, Br^{82} better than $\pm 1.5\%$. The pre-

vibration stirring with the same active Ringer solution as was used in the experiment of Fig. 42. The perfusion velocity was held constant (about 1.8 g perfusate per sec corresponding to $\bar{v} \approx 80$ cm/sec) for 15 seconds when it was stopped for 1.5 sec. Perfusion was then restarted at the same velocity which was then gradually reduced (note the rising concentrations of the isotopes in the second part of the experiment). The time courses of the effluxes (amount) of the tracers during the first perfusion period are similar to those of curve II in Fig. 42. The rest of the experiment is interpreted as follows: During the 1.5 seconds when the perfusion was stopped, outflow of the tracers into the stationary solution surrounding the preparation continued, thus the amounts of Na^{24} and Br^{82} in the perfusate, when the perfusion is recommenced, are initially high, but very soon return to about the same level as just before perfusion was stopped. The Br^{82}/Na^{24} ratio diminished as the perfusion velocity was reduced during the second half of the experiment (cf. experiment of Fig. 42). Whether there is actually a ratio change in relation to the perfusion velocity is difficult to assess, but it seems plausible. Changes in relative depletion would give a varying ratio, but this can be excluded (cf. page 97, the experiment of Fig. 42). The concentration of Na^{24} and Br^{82} in the perfusate shows that if the perfusion volume is below a certain critical level (after the time of 20 seconds) the tracer concentration rises even though the total amount in each sample is constant or diminished. *Small variations in the perfusion velocity for this reason may thus result in large fluctuations of the concentration in the perfusate.*

In the experiments of Figs. 42 and 43, the same active Ringer solution was used for loading the preparations, which facilitates certain comparisons between these experiments (further experimets are desirable). It seems probable from the experiments of Figs. 42 and 43 that the perfusion velocity is a factor affecting the rate of efflux of the tracer from the preparation. It is also probable that the nature of the ion itself is important since the ratio between the amounts of Br^{82} and Na^{24} in the perfusate were also dependent on perfusion veloc-

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paration was soaked for 1 hour at room temperature with vibration stirring in a loading solution containing 211.7 kcpm Na^{24}/mg, 187.0 kcpm Br^{82}/mg, i.e. $Br^{82}/Na^{24} = 0.86$ (1 kcpm $= 10^3$ cpm). The corresponding ESA for the active Ringer solution was 2550 kcpm Na^{24}/μmole Na and 6631 kcpm Br^{82}/μmole Br.

The figure shows the concentrations and total amounts for Na^{24} and Br^{82} in the perfusate. The volumes of perfusate samples are shown in the lower part of the figure (there were 2 samples per second) for the two efflux experiments (I and II) made on the same preparation (each after loading for 1 hour).

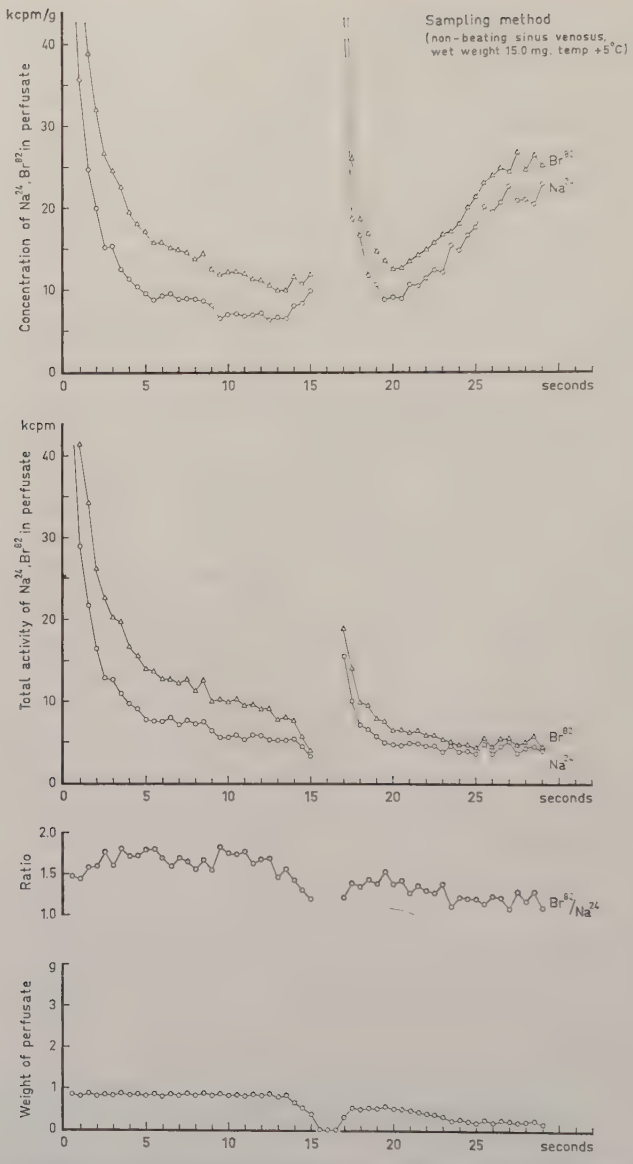
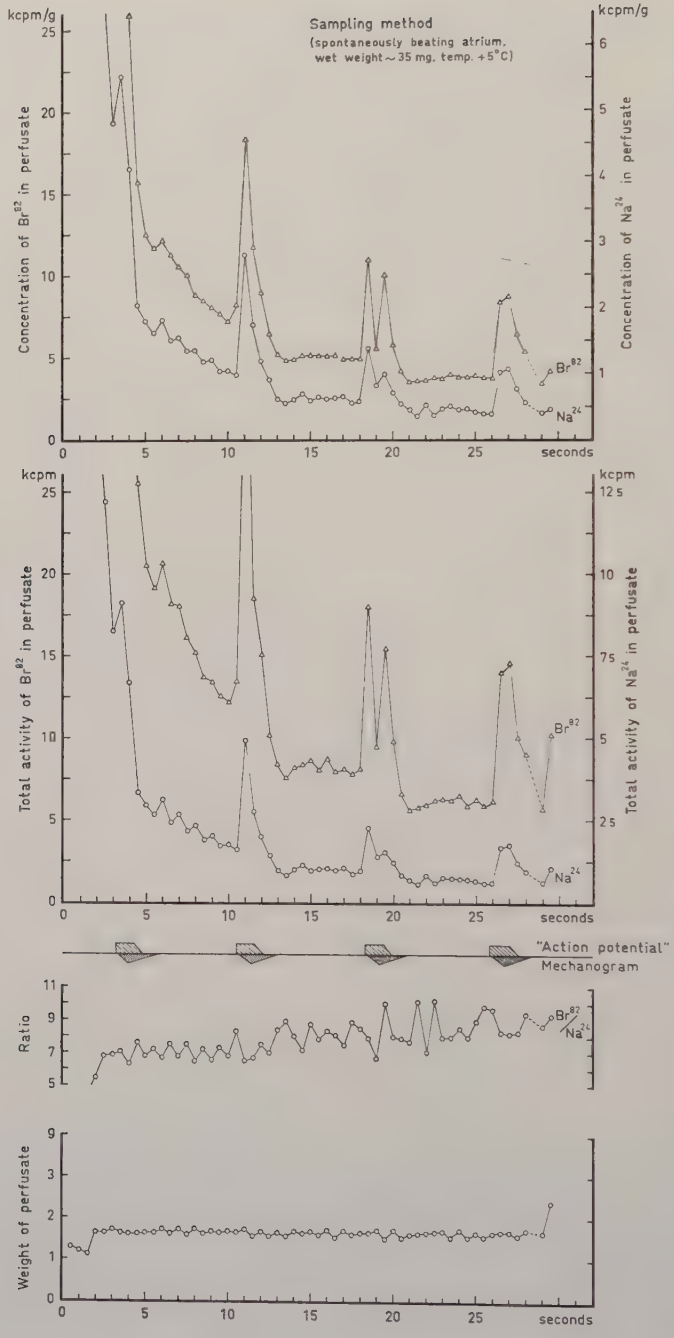


FIG. 43. Efflux of Na^{24} and Br^{82} from a non-beating sinus venosus (method described in chapter V, B, a). For accuracy of analysis see legend of Fig. 42. The preparation was loaded for 1 hour at room temperature with the same active Ringer solution as in the experiment of Fig. 42 (see figure text). The figure shows the concentrations and total amounts of Na^{24} and Br^{82} and the Br^{82}/Na^{24} ratios in the perfusate samples. The lowest curve shows the weights of the sample of perfusate (2 samples per second). Perfusion was stopped between 15 secs and 16.5 secs (1.5 secs) and then recommenced at almost the initial rate which was then progressively decreased (see text for further details).

ity. It seems most probable that the lower ratio obtained with lower perfusion rates depends essentially on a reduction in the efflux rate of Br^{82} (cf. the amounts of Br^{82} and Na^{24} in the experiment of Fig. 42 between the times 9–13 sec at both velocities; cf. also the values in the perfusate of the experiment of Fig. 43 at 10 and 24–26 sec). The variations in tracer concentration in the perfusate were greater with lower perfusion velocities. *At low perfusion velocities both the low velocity itself and the unstirred layer may become rate-limiting steps* in determining the movement of the tracer from the preparation into the perfusion solution.

When the tracer moves from the tissue into the convection stream of the non-radioactive Ringer perfusant, it has to pass through the unstirred layer surrounding the preparation. This unstirred layer can schematically be regarded as a compartment lying between the tissue and convection stream compartments. The volume of the tissue can be regarded as constant, while that of the convection stream of inactive Ringer solution depends on the perfusion velocity through the system; the greater the velocity the larger the volume. *The volume of the unstirred layer compartment depends to some extent on the perfusion velocity* (cf. chapter VII, B) in that at higher velocities it is reduced. According to Harris (1960, p. 148) the diffusion is considerably slower in the extracellular space than in free solution, because of restriction in the tissue matrix. Ionic diffusion in an unstirred layer surrounding a biological preparation may also be modified, compared with that in free solution (cf. Hale and Reichenberg, 1949; Helfferich, 1959).

The total activity ratio Br^{82}/Na^{24} which was dependent on the perfusion velocity (experiments of Figs. 42 and 43) can be interpreted in terms of the effects of an unstirred layer. As is evident in Fig. 42, and particularly in Fig. 43, the concentration of the tracer is inversely related to the perfusion velocity. At a high velocity the concentration gradient across the boundary between the unstirred layer and the convection stream of non-radioactive Ringer solution is greater than with a low perfusion velocity. At high velocities therefore the differences in mobility become more evident, because the transport processes are further away from the equilibrium state. Thus since the Br^- ion has a mobility which is about $1\frac{1}{2}$ times that of the Na^+ ion (cf. Robinson and Stokes, 1955), the Br^{82}/Na^{24} ratio should increase with increasing perfusion velocity.


Sampling method
(spontaneously beating atrium,
wet weight ~ 35 mg, temp. +5°C)
kcpm/g
Concentration of Br82 in perfusate
Concentration of Na24 in perfusate
Br82
Na24
seconds
kcpm
Total activity of Br82 in perfusate
Total activity of Na24 in perfusate
"Action potential"
Mechanogram
Ratio
Weight of perfusate

The movement of tracer from the spontaneously beating preparation to the perfusion solution at different perfusion velocities

Fig. 44 illustrates an efflux experiment on an inverted, spontaneously beating atrium mounted on an ECG catheter as described in chapter II, b. The preparation was loaded with Na^{24} and Br^{82} by soaking for $1\frac{1}{2}$ hours at room temperature with vibration stirring; it was then mounted in the perfusion chamber as shown in Fig. 22 (p. 46, sampling method). In order to obtain poor perfusion conditions the part of the perfusion chamber which normally surrounded the preparation was omitted; in this way the "perfusing" non-radioactive Ringer solution ran only over the surface of the tissue. The figure gives the concentrations as well as the total activities of Na^{24} and Br^{82} in the perfusate samples. The perfusion velocity was about 3.2 g per sec. The ratios between the total activities of Br^{82} and Na^{24} in the perfusate samples are shown in the figure. Electrical (action potential) and the mechanical (mechanogram) activities are shown schematically. As the figure shows there were cyclical variations in both the total amount and concentration of Na^{24} and Br^{82} in the perfusate samples. Electromechanical activity coincided with the efflux peaks. The Br^{82}/Na^{24} efflux ratio showed no definite cyclical variations.

A Br^{82} efflux experiment on a spontaneously beating sinus venosus is illustrated in Fig. 45. The preparation was loaded for 1 hour in active Ringer solution prior to the efflux study, which was carried out as described in chapter V, B, a (the efflux chamber was mounted as shown in Fig. 22, p. 46). Fig. 45 shows both the concentration and total amount of Br^{82} in the perfusate samples; the perfusion velocity was about 3 g per sec, corresponding to $\bar{v} \approx 130$ cm/sec. The electrical and mechanical activities are shown schematically as in Fig. 44. It seems likely that there is some correlation between increases in concentration and total activity of Br^{82} in the perfusate and the electromechanical activity.

Fig. 44. Efflux of Na^{24} and Br^{82} from a spontaneously beating atrium (see chapter V, B, a). Accuracy of analysis was for $Na^{24} \pm 2.5\%$ and for $Br^{82} \pm 1.5\%$. The atrium was loaded for $1\frac{1}{2}$ hours at room temperature with vibration stirring in active solution containing 15.5 kcpm Na^{24}/mg and 67.0 kcpm Br^{82}/mg, i.e. $Br^{82}/Na^{24} = 4.34$. The ESA values were: 186 kcpm Na^{24}/μmole Na and 2377 kcpm Br^{82}/μmole Br (1 kcpm $= 10^3$ cpm).

The figure shows the concentrations and total amounts of Na^{24} and Br^{82} in the perfusate samples (sample volumes shown below; 2 samples per second). The electrical and mechanical activities are shown schematically (see also text).

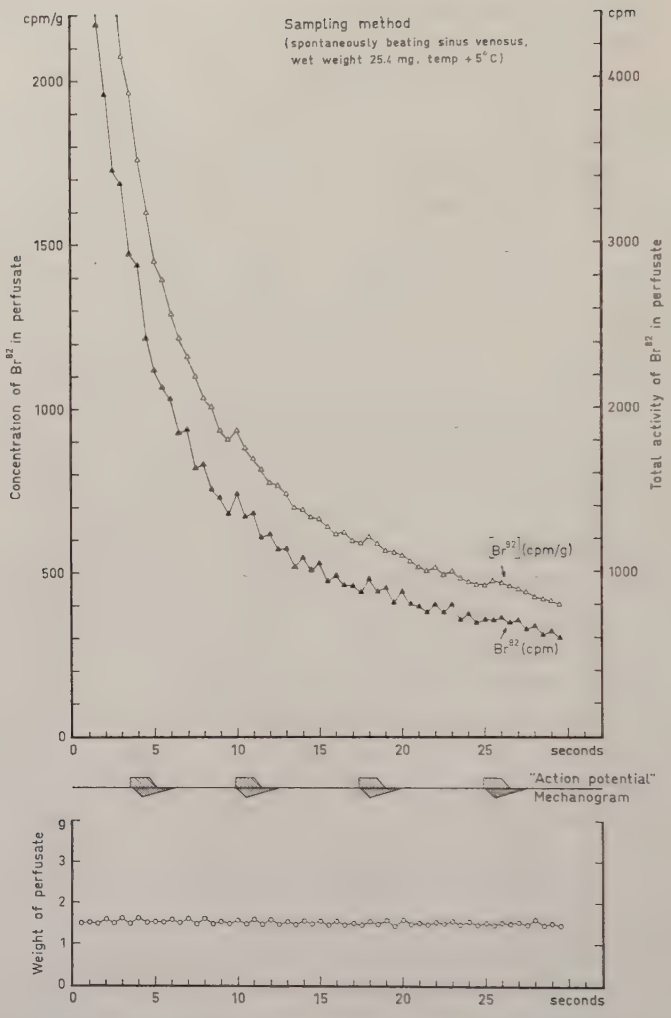


FIG. 45. Efflux of Br^{82} from a spontaneously beating sinus venosus (chapter V, B, a). Accuracy of analysis was ± 1 %. The sinus venosus was loaded for 1 hour at room temperature with vibration stirring in an active Ringer solution containing 11.6 kcpm Br^{82}/mg (ESA = 412 kcpm Br^{82}/μmole Br), (1 kcpm = 10^3 cpm). The figure shows the concentration (left ordinate) and total amount (right ordinate) for Br^{82} in the perfusate samples (sample volume given below; 2 samples per second). The electrical and mechanical activities are shown schematically (see also text).

The mechanical activity, as discussed in chapter VII, F, *may affect both the observed influxes and effluxes by producing variations in the surface volume relationships.* Such variation could give rise to mechanical artifacts in two ways: 1) "*Extracellular squeezing*" *resulting in expulsion of extracellular fluid into the perfusion solution* may occur. A "squeezing" effect might also alter the dimensions of the small extracellular spaces and in this way affect the physical chemical properties, e.g. for diffusion. 2) *The changing form of the preparation during contraction may distort the extra-cardiac surface layer.* In an efflux experiment this surface layer corresponds to an unstirred layer, which thus may undergo variations during the mechanical cycle. The only certain way to exclude these possibilities would be to study simultaneous fluxes of different tracer ions, of which one could be safely assumed to be essentially extracellular. The SO_4^- ion fulfils the later role, the heart muscle cells are relatively impermeable to it (cf. Johnson, 1955; Page, 1962; Niedergerke, 1963; Danielson, 1964). This ion can also be obtained with high specific activity of S^{35}.

An efflux experiment for Na^{24}, Br^{82} and $S^{35}O_4$ was made on a spontaneously beating sinus venosus (see Fig. 46*a*) with a perfusion velocity corresponding to that used in the influx experiments by the DMM.[1] At this perfusion velocity (7 g of perfusion solution per sec corresponding to $\bar{v} \approx 300$ cm/sec) the extra-cardiac unstirred layer is very small and not likely to be rate-limiting for the movement of tracer between the preparation and the perfusion solution (cf. the summary of chapter VII, B). As can be seen in Fig. 46*a* there were no certain variations in the total activity or concentration of Na^{24}, Br^{82} or $S^{35}O_4$ in the perfusate correlated with the electro-mechanical cycle. It is evident further that variations in the efflux of the three isotopes are clearly parallel.

In order to study the effect of the mechanical activity an efflux experiment was made on the same preparation as in Fig. 46a after soaking for 1 hour in radioactive Ringer solution containing only 0.5 mM Ca^{++} (the specific activity of Na^{24}, Br^{82} and $S^{35}O_4$ in the Ringer solution was the same as in the experiment of Fig. 46*a*). *The contrac-*

[1] Na^{24} and Br^{82} were determined by gamma spectrometry as described in chapter IV, C, a, 2. The 0.167 MeV beta emission of the S^{35} (no gamma emission) was absorbed by the aluminium sheath of the NaI(Tl) crystal. S^{35} was determined, after decay of the Na^{24} and Br^{82}, by determination in a liquid scintillation counter as described in chapter IV, C, b.

tion was reduced to about 25% of that with 1.8 mM Ca^{++} (cf. chapter VII, F). There were no variations in either the concentration or total activities of the three isotopes in the perfusate which could be correlated with the electro-mechanical activity, as is evident from Fig. 46*b* (illustrates only the concentration of isotope in the perfusate). In this case there were greater fluctuations in the efflux and clear parallelism between the behaviour of the three isotopes.—*After soaking for 1 hour in radioactive Ringer solution with 1.8 mM Ca^{++} the mechanical activity had returned to the level of the experiment shown in Fig. 46a* (cf. Fig. 41, p. 41). The result of this efflux experiment is shown in Fig. 46*c*.[1]— In the experiments of Figs. 46*a–c* there were no clear AC variations in the efflux pattern with normal and reduced mechanical activity (cf. particularly Figs. 46*b* and 46*c*, where the mean curves for the concentration in the perfusate of the individual isotopes have a practically identical course). *The efflux of Na^{24}, Br^{82} and $S^{35}O_4$ is thus independent of the mechanical activity of the preparation* (at 100% and 25% of the normal contraction amplitude) *and also of any "extracellular squeezing" if this occurs. No definite connection between the mechanical activity of the tissue and the tracer flux was observed* in the experiments shown in Figs. 46*a–c*. If such variations did actually occur they were masked by other irregular fluctuations [e.g. the variability of the flux itself, instrumental errors and

[1] The decrease in spontaneous frequency with a low Ca^{++} environment which was discussed in chapter VII, F can be clearly seen in Fig. 46 *b* in comparison with Figs. 46 *a* and *c*.

FIG. 46*a–c*. Three successive efflux experiments for Na^{24}, Br^{82} and $S^{35}O_4$ on a spontaneously beating sinus venosus (see chapter V, B, a). Accuracy of analysis was for $Na^{24} \pm 2.5\%$, $Br^{82} \pm 2.0\%$ and $S^{35} \pm 1.5\%$. Before each experiment the preparation was loaded for 1 hour in an active Ringer solution containing 15.7 kcpm Na^{24}/mg, 23.2 kcpm Br^{82}/mg and 21.5 kcpm S^{35}/mg (ESA: 184 kcpm Na^{24}/μmole Na, 1657 kcpm Br^{82}/μmole Br and 21,530 kcpm S^{35}/μmole SO_4), (1 kcpm = 10^3 cpm).

The figure shows in all cases concentrations. Fig. 46*a* shows in addition the total amounts of Na^{24}, Br^{82} and $S^{35}O_4$ in the perfusate samples (sample volumes given below; 2 samples per second). Electrical and mechanical activities are shown schematically.

Fig. 46*a* illustrates the first efflux experiment after soaking in Ringer solution with 1.8 mM Ca^{++} (100% isometric contraction amplitude); Fig. 46*b* shows the second experiment after soaking in only 0.5 mM Ca^{++} (25% contraction amplitude) and Fig. 46*c* the third experiment after soaking again in the Ringer solution containing 1.8 mM Ca^{++} (100% contraction amplitude). After the third experiment (Fig. 46*c*) the total amounts of Na^{24} and Br^{82} of the preparation were 136.28 kcpm and 285.78 kcpm respectively. Reference should be made to chapter VIII, F.

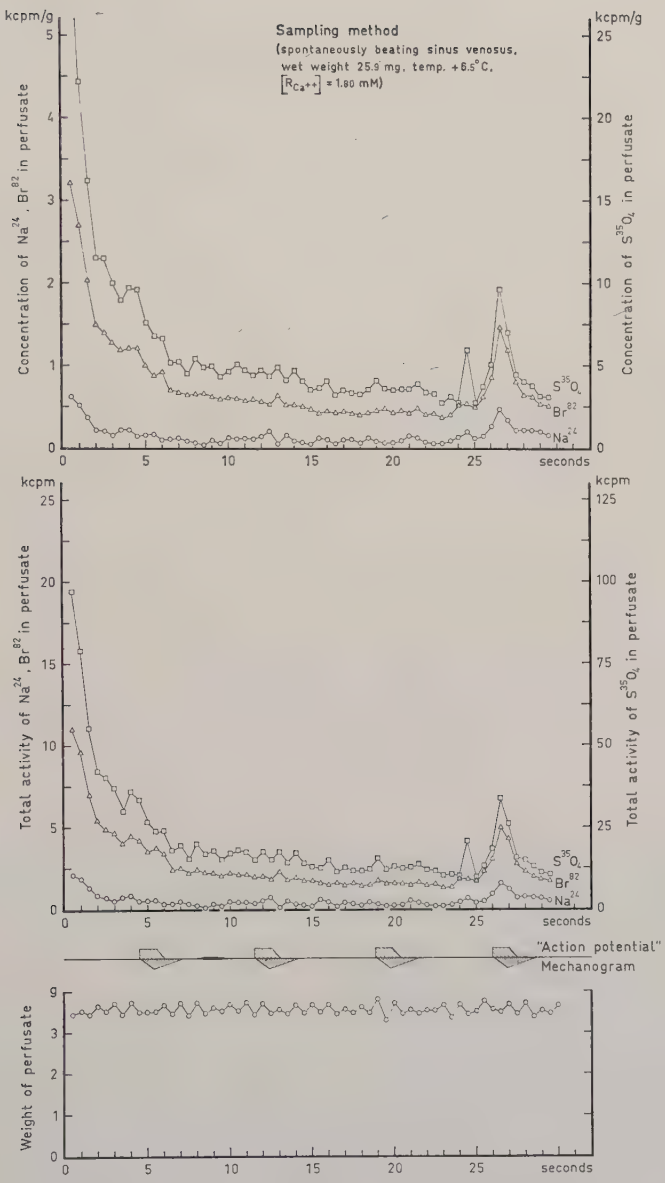

Sampling method
(spontaneously beating sinus venosus,
wet weight 25.9 mg, temp. +6.5°C,
[R_Ca++] = 1.80 mM)
kcpm/g
Concentration of Na24, Br82 in perfusate
Concentration of S35O4 in perfusate
S35O4
Br82
Na24
seconds
kcpm
Total activity of Na24, Br82 in perfusate
Total activity of S35O4 in perfusate
"Action potential"
Mechanogram
Weight of perfusate
g

Fig. 46 *a*.

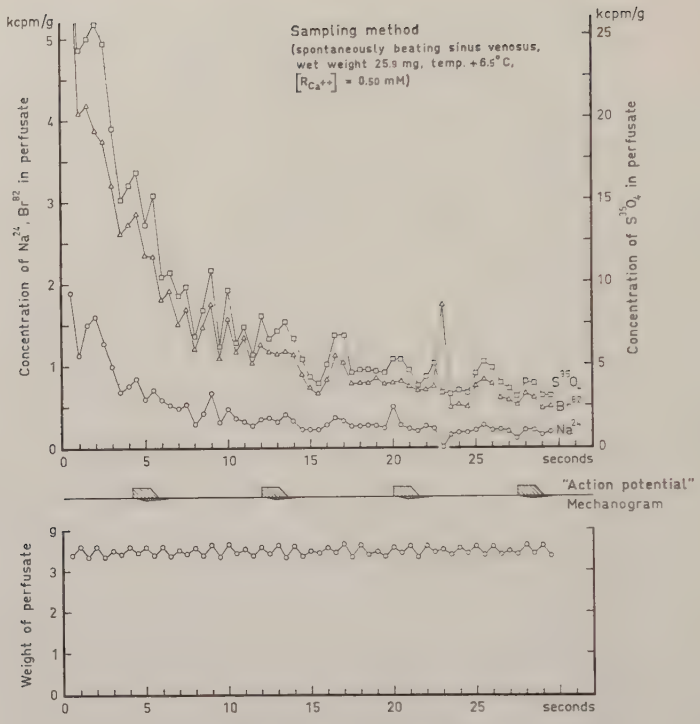


Fig. 46*b* (legend p. 106).

statistical counting error (cf. chapter VI, p. 53)]. The result is in agreement with other efflux experiments for Na^{24} and Br^{82} on spontaneously beating sinus venosus preparations with perfusion velocities corresponding to that used in the influx experiments (DMM), i.e. a mean linear velocity of 300 cm/sec.

If "extracellular squeezing" were an important factor in determining the efflux pattern, this should also be observed in an experiment when the perfusion conditions are comparable to those in the experiments of Fig. 46a–c. In these experiments the extra-cardiac unstirred layer is presumably small, and contact between the preparation and the perfusion solution particularly good.

Fig. 46*a–c* show also one of the *weaknesses of the efflux method.* Only four cardiac cycles could be studied (longer efflux experiments have also been made, but have not essentially augmented our knowledge), which together with the condition that the specific activity

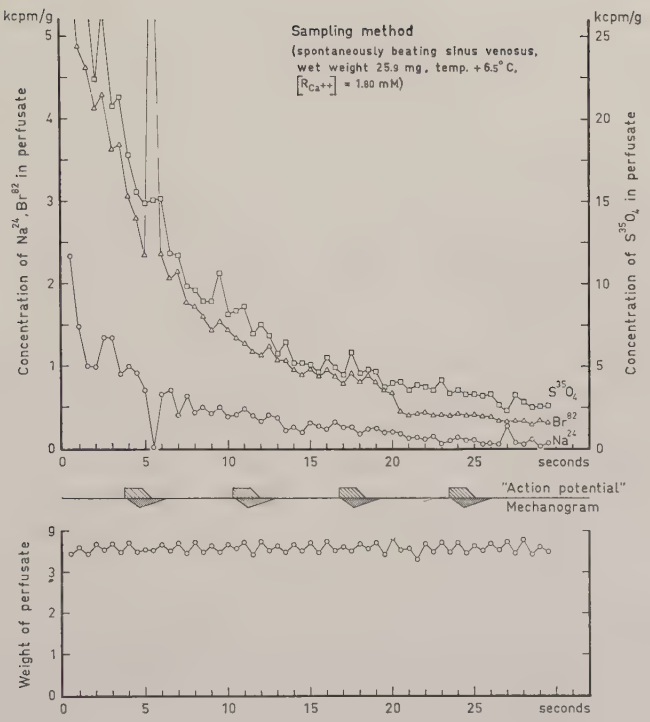


Fig. 46 *c* (legend p. 106).

of the preparation is in a transient state make it *impossible to use a type of continuous averaging in order to eliminate the background noise.*

Only in those cases where the perfusion conditions could be regarded as poor were the variations of total activity and concentrations of the isotope in the perfusate correlated with the electro-mechanical events. This suggests that *the mechanical activity causes variations in the external unstirred layer, which at low mean linear perfusion velocities becomes an important buffer compartment between the tissue and the inactive Ringer solution.* Efflux of tracer occurs continually from the tissue into this buffer compartment, and if it varies in size in relation to the mechanical cycle (changed surface volume relationship) so that, for example, its volume is reduced with a contraction, this will be manifested in the perfusate as an increased quantity and concentration of tracer. *It is unlikely that such small volume fluctuations could be recorded even by weighing the perfusate or using a*

radioactive volume indicator. At higher perfusion velocities when the extra-cardiac unstirred layer is reduced, the effect of the mechanical activity is also presumably greatly reduced.

Summary: Efflux of tracer from the tissue to the convection stream perfusing non-radioactive Ringer solution must occur through the unstirred layer which surrounds the preparation under these conditions. With a low perfusion velocity it seems likely that an unstirred layer may be a rate-limiting step for the efflux of tracer from the preparation. Evidence was obtained suggesting that the perfusion velocity affected the efflux kinetics and that different ions were affected quantitatively differently. In the efflux experiments with spontaneously beating preparations, it is only when the perfusion conditions could be described as poor that there were variations in the total activity and concentrations of the isotopes which could be correlated to the electro-mechanical cycles. *If an "extracellular squeezing" were an important determinant of efflux kinetics, this effect should also be observed when the perfusion conditions are comparable to those in an influx (DMM) experiment (Fig. 46a–c). The results obtained suggest rather that distortions in the extra-cardiac surface layer occur; if these become evident in the efflux pattern, they would be expected to be correlated with the mechanical activity.* See further general discussion.

As discussed in chapter I there are only a few reports in the literature of direct studies of the time relation between ion fluxes and the electro-mechanical activity of the heart tissue. WILDE and O'BRIEN (1953), DANIELSON, ÖBRINK and SJÖSTRAND (1961, 1962) and LORBER *et al.* (1962) studied the K^{42} efflux from isolated heart preparations ("wash-out" technique). DANIELSON, ÖBRINK and SJÖSTRAND (1961, 1962) studied also Na^{24} efflux. Preliminary results on the Na^{24} influx (sinus venosus) have been reported by SJÖSTRAND (1962, 1963).

In the present work the efflux from spontaneously beating sinus venosus and atrium has been studied with the help of radioactive isotopes with a "wash-out" technique. The radioactive influx has also been studied with a new perfusion and analysis technique, referred to previously as the "direct monitoring method" (DMM). By perfusion with a high linear mean velocity ($\bar{v} \approx 300$ cm/sec) the efflux of Na^{24} and Br^{82} did not exhibit any fluctuations which could with certainty be correlated with the electro-mechanical cycles of activity of the spontaneously beating preparation. In influx experiments made by the DMM fluctuations related to the electro-mechanical events of the heart cycle were obtained for both Na^{24} and Br^{82}. The methodological problems associated with these experiments were discussed in chapters VI, VII and VIII.

The complexity of the tissue preparation used

An isolated heart muscle preparation is both structurally and functionally a particularly complex system. It is multi-cellular and consists structurally of several tissue compartments (a classification into only intracellular and extracellular spaces appears in practice to be an over-simplification). From a functional aspect also, the concept of only two compartments, intra- and extracellular, appears to be insufficient. The heart muscle cell differs from the much studied nerve axon in having in addition to the resting and action potentials a mechanical activity. It is not easy to dissociate the electrical activity (action potential) and the mechanical activity (contraction), which

run rather in parallel. Studies of a tracer flux in a preparation with such a complex structural and functional constitution cannot *a priori* be expected to give results which indicate a simple ion flux pattern in terms of only intra- and extracellular compartments.[1]

General views regarding tracer measurements on heart tissue

The use of radioactive isotopes in the determination in the resting state of the ionic movements between the intra- and extracellular spaces presents varying degrees of difficulty. A measured tracer flux can in general be said to depend on at least two main factors, the exchange relationships between the cells and the extracellular space, and between the latter and the perfusion solution. Measurements of the K^+ flux are therefore the simplest, since the intracellular concentration of this ion is high. The difficulties involved in the determination of Na^+ and Cl^- (Br^-) fluxes are considerably greater, since their intracellular concentrations are rather low. The determination of the sodium exchange between cells and extracellular space (and probably also Cl^- exchange) seems at present possible only by using extremely short time constants. Even if the analysis technique can give, from the time aspect, good resolution, the results obtained are difficult to assess if damping phenomena and time lags in the system studied cannot be calculated accurately (the extracellular volume should for these reasons be small compared to the intracellular compartment).

The temperature effect on electrolyte distribution and fluxes must also be considered. Haas and Glitsch (1962) found that the K^{42} efflux from non-beating frog atria increased with temperature. Studying the temperature effect on electrolyte exchange in cat heart papillary muscle Page, Goerke and Storm (1964) found no changes in cell K and Na contents in mammalian papillary muscles cooled to 17–18°C; with further cooling there were marked losses of cell K and increases in cell Na, Cl and water. However, cooling of frog heart muscle from room temperature to + 5°C is not analogous to cooling mammalian heart tissue to a similar extent in relation to the "normal" temperature (cf. the results for K^{42} efflux from frog atria by Haas and Glitsch cited above).

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[1] The kinetics of ion exchange between different phases in a multicompartment system have been treated by Haas (1962).

Possible mechanical influences on tracer kinetics in "wash-out" experiments on heart muscle

Any effects on the tracer flux induced by mechanical activity would most likely be correlated with the contraction cycles. For instance it seems not unlikely that the surface volume relationships of the tissue preparation will be altered during mechanical changes. As pointed out in chapter VIII, mechanical "squeezing" may have two main effects, 1) the expulsion of extracellular fluid, and 2) mechanical distortion of an unstirred layer surrounding the preparation during perfusion ("external squeezing" effect). The results reported in chapter VIII do not exclude the possibility of an "extracellular squeezing", and it is probable that under certain perfusion conditions an "external squeezing" effect becomes a limiting factor. It must therefore be borne in mind that when ion effluxes as studied by the "wash-out" technique appear to be correlated with the events of the heart cycle the question of an "external squeezing" effect should be excluded.

The release of K^{42} from a perfused turtle heart

By perfusing the coronary system of a K^{42} loaded turtle heart, Wilde and O'Brien (1953) (see also Wilde, 1957*a*, *b*) found cyclical variations of the K^{42} efflux (correction for the transport time and boundary spreading due to the long distance from the capillaries in the preparation to the point where the perfusate was collected were made). The perfusion volume was about 0.03 ml per second, and was determined directly by adding I^{131} labelled albumin or $P^{32}O_4$ (the flow was constant between contractions, but during systole a "squeezing" effect occurred, the volume collected increasing three-fold; the authors believed that this was due to the veins being emptied as when a sponge is squeezed). A corrected K^{42} concentration was calculated by adjustment for volume changes in flow; these values exhibited a maximum immediately after the T-wave in the electrocardiogram.— It should be pointed out that the perfusion volume in these experiments was small, subject to large fluctuations. With the technique used for the determination of the perfusion volume, corrections for volume fluctuations which might be caused by changes in the intravascular buffer volume, induced by the mechanical activity could not be calculated. The intravascular buffer volume can be conceived as an unstirred layer (corresponding to the immobile layer in the

plasmatic zone, discussed by Copley, Scott Blair, Baléa and Staple, 1960), which could almost certainly be affected by the changing perfusion flow in the vascular system (cf. chapter VIII, p. 99).

Efflux of Na^{24} and K^{42} from the isolated frog heart

Danielson, Öbrink and Sjöstrand (1961, 1962) found cyclical variations of Na^{24} efflux when an isolated frog heart preparation (sinus venosus, atrium or sinus atrium) was perfused. Because of the difficulty of obtaining sufficiently high specific activities of K^{42}, only in one efflux experiment on the sinoatrial preparation was a sufficiently high activity of K^{42} obtained. Movements of Na^{24} and K^{42} studied simultaneously showed cyclical varitions in relation to the recorded electrical activity (the ratio between Na^{24} and K^{42} in the perfusate did not show any definite variations in relation to the heart cycle). The perfusion velocity was between 0.1 and 0.5 ml/sec (variations of $\pm 10\,\%$), corresponding to a mean linear velocity of $\bar{v} \approx 5$–22 cm/sec. The perfusion flow should therefore have been within the region where the tracer efflux should be expected to show large concentration variations in response to small changes in the perfusion velocity (cf. Figs. 42, p. 98 and 43, p. 100).

Efflux of K^{42} from frog ventricle

Lorber *et al.* (1962) perfused a frog ventricle preparation and found cyclical variations in the total amount of K^{42} in the perfusate (there was a maximum during and of approximately of the same length as the action potential). The possibility that the efflux pattern observed was caused by mechanical activity was excluded by the authors because of the change in the appearance of the efflux peak (lower and broader) when the perfusing fluid was changed from normal to potassium-free Ringer. An immediate and marked increase of the isometric tension occurred and lasted for several minutes when the normal Ringer was replaced by the potassium-free solution; the authors considered that reduced contractility would alter the form of the efflux peaks for mechanical reasons. On the other hand, perfusion with potassium-free Ringer solution resulted in a marked decrease in the efflux rate for K^{42}. The perfusion velocity in these experiments was about 0.17 ml/sec (corresponding to a turn over of 20 times per second of the free fluid volume surrounding the tissue in the perfusion chamber).—From the data given it can be calculated that the

mean linear perfusion velocity was not greater than 50 cm/sec, and thus the conditions in comparison with those in the system used in the experiments reported in chapter VIII are most nearly comparable with those in the experiment of Fig. 42, p. 98 (experiment I), Fig. 43, p. 100 (first period of efflux experiment), and Fig. 45 (p. 104). It seems therefore likely that in the experiments of Lorber *et al.* (1962) there was a significant extra-cardiac buffer volume, and therefore an "external squeezing" effect is a possible alternative explanation for the K^{42} pattern they obtained. The increased isometric tension after change over to a potassium-free Ringer solution does not exclude an "external squeezing" effect, since the total efflux of K^{42} was strongly reduced at the same time. The movements of K^{42} across the external surface layer could therefore be expected to be considerably slower; the concentration of K^{42} in this layer would therefore be estimated to be lower on perfusion with potassium-free Ringer solution, whereby in spite of increased mechanical activity the efflux peak for K^{42} would be reduced.

Direct monitoring method (DMM)—advantages and limitations

As pointed out in chapter VIII, one of the weaknesses with the "wash-out" technique is that continuous averaging cannot be performed. With an influx experiment according to the direct monitoring method (DMM, see p. 45), the conditions are considerably more favourable for an analysis of the tracer flux, because continuous averaging is possible. This means that although individual measurements may be regarded as uncertain, and even exhibit such small variations as to lie within the error of the method, they can collectively be used to derive useful information (continuous averaging is discussed in chapter VI). *In the DMM continuous averaging over a large number of cycles may reveal systematic variations in the tracer flux.* If the perfusion conditions during the whole experiment are the same (constant relationships between the lengths of the Ringer and silicone oil perfusion periods) the variations in the analytical and perfusion systems will have the character of "random fluctuation noise".

DMM also has a rather good time resolution. It was pointed out in chapter VII, C (p. 83) that theoretically the mean cycle obtained by the continuous averaging technique can exhibit a time shift

corresponding maximally to $-(t_r+t_s)$ msec in relation to the reference point (=e.g. the ECG recording), and thus the correction range is known. The resolution with respect to time in the DMM is therefore determined by how short t_r (the Ringer perfusion period) and t_s (silicone perfusion period) can be made; these are technical matters.[1]

If the DMM is made when a uniform specific activity has been obtained (the specific activity of the preparation = that of the perfusing active Ringer solution), it offers advantageous conditions for studying net fluxes between the preparation and the perfusing solution. What information these net fluxes can give depends on the properties of the object studied; experiments on heart muscle preparations must give less clear results than studies of non-contractile preparations. A tissue consisting of a single cell or cell groups without an extracellular space (tissue culture) also makes it easier for direct studies of any transmembrane net fluxes. It should, however, be pointed out that DMM can only give information about the flux of the tracer into the preparation as a whole (amount per unit time) and so long as there are internal concentration gradients for the specific activity (uneven specific activity) estimates of the flux in pmole/cm^2 or mmole/kg of tissue per beat cannot be made.

General comments on the influx experiments (DMM) performed

The experiments so far performed with DMM have been made essentially to demonstrate the technique and to investigate its potentialities. The resolution in the analysis method (signal/noise ratio) can in general be expected to be greatest when the signal/noise ratio is highest; since the background is much greater than the signal in the preparation, the resolution is rather poor. It is therefore advantageous to study the first part of the tracer influx (upstroke of the DC component) even if the later part (plateau of the DC component) could be expected to better reflect the tracer influx into the cellular space.[2] Only in one of the influx experiments so far performed by

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[1] In the experiments made with the DMM up to now, t_r was ≈ 100 msec and $t_s \approx 1000$–1500 msec.

[2] As pointed out earlier (p. 85) it can be expected that there are several components in the tracer influx (a rapid fraction corresponding to the extracellular space, and one or more less rapid components probably corresponding to the intracellular space; see further chapter VII, D, p. 84).

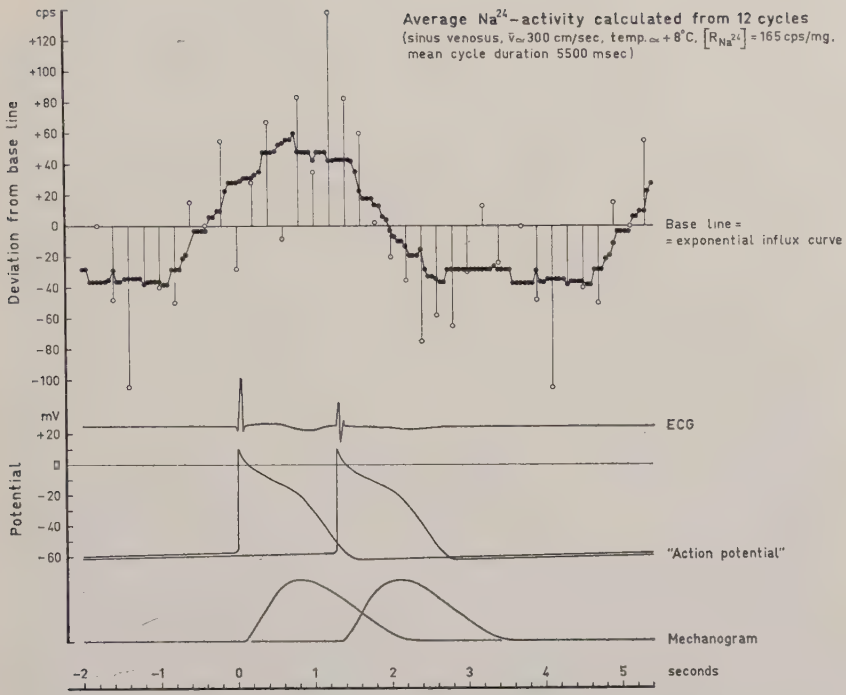

FIG. 47. A mean cycle for Na^{24} activity calculated by the *c-a-t* method for the first influx experiment of Fig. 24 (p. 52). The base-line corresponds to the mean trend line (described in chapter VI, p. 54). The ECG is drawn from the original recording while the action potential and mechanogram are drawn from other experiments on the same type of preparation (chapter II, c, p. 17). The open circles are the medians of the deviations from the base line of the original measurements occurring in each 200 msec interval ('original value method'). The solid circles are the medians of 12 intercept-interpolations values for each 50 msec interval ('intercept-interpolation method'). The standard deviation for the mean values are similar to those given for the experiment of Fig. 26 (p. 56) in chapter VI (p. 57).

The maximal uncertainty in the positions of the radioactivity pattern in relation to electromechanical activity is $(t_r + t_s)$. In this case a shift of $(t_r + t_s) = (105 + 1195) = 1300$ msec to the left. (Each measurement is arbitrarily assigned to the end of the period whose radioactivity is measured; the most correct value must therefore lie somewhere within the interval of this period, i.e. not more than $(t_r + t_s)$ to the left of where it is arbitrarily placed. Cf. p. 83.) Wet weight of preparation 19.3 mg. Calculated $O_R = 1.6$ mg; calculated "trapped volume" = 0.7 mg. Perfusion and analysis programme (DMM): $t_r = 105$ msec, $t_s = 1195$ msec, $t_m = 928$ msec. Active Ringer solution ESA 1.48 kcps Na^{24}/μmole Na (solution I), (kcps = 10^3 cps).

DMM was an equilibrial level obtained (the experiment of Fig. 25, p. I). In calculating the mean cycle the relation of the AC component to that of the DC component was calculated during a period when the DC component was not steady, since a uniform specific

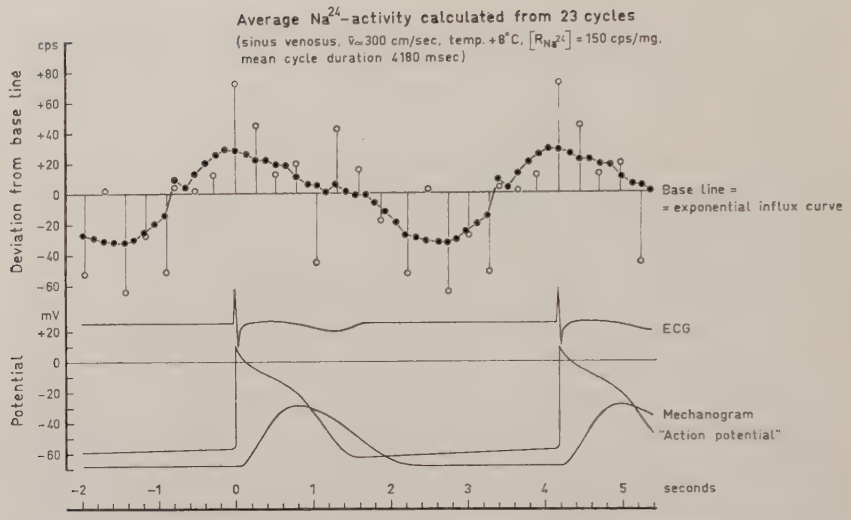

FIG. 48. A mean cycle for Na^{24} activity calculated by the *c-a-t* method from 23 cycles in an influx experiment (DMM) on a spontaneously beating sinus venosus. The base-line corresponds to the mean trend line (described in chapter VI, p. 54). The ECG is drawn from the original recording while the action potential and mechanogram are drawn from other experiments on the same type of preparation (chapter II, c, p. 17). The open circles are the arithmetic means of the deviations from the base line of the original measurements occurring in each 260 msec interval ('original value method'). The solid circles are the arithmetic means of 23 intercept–interpolation values for each 130 msec interval ('intercept–interpolation method'). The standard deviation for the mean values is similar to that given for the experiment of Fig. 26 (p. 56) in chapter VI (p. 57).

The maximal uncertainty in the positions of the radioactivity pattern in relation to electromechanical activity is in this case a shift of $(t_r + t_s) = (120 + 1195) = 1315$ msec to the left (cf. legend Fig. 47). Wet weight of preparation 19.7 mg. Calculated $O_R = 1.7$ mg; calculated trapped volume = 0.7 mg. Perfusion and analysis programme (DMM): $t_r = 120$ msec, $t_s = 1195$ msec, $t_m = 1020$ msec. Active Ringer solution ESA 1.35 kcps Na^{24}/μmole Na (solution I). (kcps = 10^3 cps).

activity had not been attained. Whether it is correct under these conditions to determine a mean cycle can naturally be discussed (cf. above). As mentioned in chapter VII, C (p. 75) it seemed permissible for technical reasons to calculate a mean cycle during the rising phase of the DC curve, because the relationship between the *mean influxes* during the Ringer and silicone oil perfusion periods remained constant for the whole experiment.

Two mean cycles for Na^{24}

With the reservations discussed above, the mean cycle for two influx experiments with Na^{24} was calculated by the *c-a-t* method

described in chapter VI. Fig. 47 shows the recorded counted activity in relation to the base line (DC component = passive distribution plus activity base line) during the first of the two influx experiments illustrated in Fig. 24 (for further information see chapter VI, p. 51). Fig. 48 shows the counting activity in relation to the base line for Na^{24} calculated for 23 heart cycles during another influx experiment. These two experiments for Na^{24}, together with a Br^{82} experiment, whose mean cycle is shown in Fig. 26 (p. 56), were from a technical aspect the most satisfactory up to now.[1] The K^{42} influx during the single heart cycle has not been studied because of the difficulty of obtaining sufficiently high specific activity of this isotope (cf. p. 50). The sinus venosus preparation on which the result shown in Fig. 47 was obtained with DMM had two electrical activities; it cannot be assessed which of these two electro-mechanical activities was the dominant (visual inspection revealed that the preparation did exhibit a double contraction).

General remarks on the Na^{24} and Br^{82} activity curves for three different heart preparations

From the results presented in Fig. 26 (Br^{82}, p. 56) and Figs. 47 and 48 (Na^{24}), it appears that the AC component in the influx experiment (DMM) on the spontaneously beating sinus venosus does contain systematic fluctuations which can be demonstrated with the *c-a-t* procedure. The mean cycles shown in these figures exhibit fluctuations in counting activity which are related to the electro-mechanical activity. A maximum of the recorded radioactivity for both Na^{24} and Br^{82} occurs together with or shortly after depolarization, while a minimum is obtained during the diastolic phase. When the slope of the activity curve for the mean cycle is positive, this means a net increase of activity in the system, and vice versa (when the slope is zero, i.e. parallel with the base line, the net flux is also zero). As pointed out in chapter VII, C, the systematic fluctuations about the exponential influx curve (base line) in a spontaneously beating preparation are

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[1] After the Br^{82} influx experiment of Figs. 25 and 26, an influx experiment for Na^{24} was made on the same preparation; this, however, failed for technical reasons (the background activity was not held constant during the experiment). The mean cycle for Na^{24} and Br^{82}, calculated from results on the same preparation, can therefore not be given.

not necessarily evidence of ionic movements in or out of cells (cf. also chapter VIII, p. 105). The fluctuations may have other causes: 1) The pattern of radioactivity in the tissue may depend on the relationship between the volume of the extra-cardiac surface film (O_R) during the silicone oil perfusion and mechanical activity. 2) The mechanical activity may cause alterations in the extracellular movements of the isotope in the tissue.—If the variations in O_R were the *sole* cause of the fluctuations in activity calculated with the *c-a-t* method, the pattern for *all* isotopes measured with the DMM should have essentially the same form and relation to the electro-mechanical cycle, independently of the relationship between t_r and t_s (this is discussed in chapter VII, C, pp. 81, 83). If the electro-mechanical activity affected the movements of the isotope within the tissue, this effect should be dependent to some extent on the relationship between t_r and t_s (cf. chapter VII, C, pp. 83, 84). As discussed in chapter VII, C (p. 81), the limits for the correlation in time between the recorded electrical activity (ECG) and the calculated radioactivity pattern for the mean cycle can be determined: One of the limits is shown in Figs. 26, 47 and 48, the other limit is maximally a shift ($t_r - t_s$) msec to the left of the radioactivity pattern in relation to the electro-mechanical cycle.

Can the tracer patterns for the "mean cycle" be explained by a change in O_R due to mechanical events?

If there is a correlation between the volume of the extra-cardiac surface film O_R during silicone oil perfusion and mechanical activity, then according to the arguments presented above the activity patterns should be the same for all isotopes (up to now only Na^{24} and Br^{82}) studied with DMM. In all cases the activity patterns should be essentially similar in form and relation to the electro-mechanical cycle; this is not the case. From the results described in chapter VIII concerning the efflux of Na^{24} and Br^{82} from a spontaneously beating preparation, it is evident that distortion in a surface layer ("external squeezing" effect) is an important factor; these variations occur in relation to the electro-mechanical activity (maximal "squeezing" effect during the "electro-mechanical activity"). If the volume of O_R was correlated to the electro-mechanical activity, it would therefore probably have a minimum during contraction, and the recorded radioactivity pattern

for the mean cycle should therefore in this case show a minimum in connection with the electro-mechanical activity. This is not the case (cf. Fig. 26, p. 56, and 48). It is difficult to attribute the results for the Na^{24} activity pattern in Fig. 47 to the mechanical activity, since the preparation exhibited a double contraction cycle. *It appears therefore that variations in O_R correlated with the electro-mechanical activity can be excluded as a cause of the alternating mean cycle radioactivity pattern calculated by the c-a-t method* (cf. chapter VII, C, pp. 81, 83).

Can the tracer patterns for the "mean cycle" be explained by a mechanical distortion of the extracellular matrix ("extracellular squeezing" effect)?

If the activity pattern depends on a correlation between the tracer kinetics in the tissue and the electro-mechanical activity, the question of an "extracellular squeezing" must be discussed. "Extracellular squeezing" may result in expulsion of extracellular fluid into the perfusion solution. Such an effect will be expected to give a reduced radioactivity in the preparation during the mechanical activity. Even if the radioactivity pattern in Figs. 26, 47 and 48 (see particularly Fig. 26), is shifted (t_r+t_s) msec to the left, *it is difficult to accept that the relationship of the radioactivity pattern to the mechanical activity can be explained as a result of "extracellular squeezing"* (cf. also the summary of chapter VIII).

The possibility of there being a true biological explanation for the pattern obtained

If it can be excluded that the radioactive pattern of the mean cycle is not wholly mechanical in origin, the possibility remains that both Na^{24} (Figs. 47, 48) and Br^{82} (Fig. 26, p. 56) oscillations arise from biological causes.[1] More refined methods, for example simultaneous studies of Na^{24} and Br^{82} on the same preparation, should give more reliable information about this matter.

As pointed out earlier, the possibility of determining a transmembrane flux in a sinus venosus is very small because of the unfavour-

[1] The only way to exclude mechanical "squeezing" with certainty would be to do an experiment with DMM on a preparation with no mechanical activity (a reduced mechanical activity can be obtained on sinus venosus, see chapter VII, F).

able relationship between the volumes of the extracellular and intracellular compartments[1] and the great disparity in the electrolyte distribution. *It must, however, be pointed out that contact between the perfusion solution and the outermost cells of the preparation is particularly good under the conditions of a perfusion experiment by the DMM.* An example of this are the K^+ depolarization experiments described in chapter VII, E. *It may therefore be possible that an ion flux between the extracellular and intracellular spaces was recorded since the measuring system used had a particularly good resolution with respect to time* (see above).

Even if it would be somewhat premature to attempt to analyse the present results in terms of current theories concerning ion fluxes and their relation to single electrical activity cycles, possible general mechanisms for the genesis of the results obtained may be considered:

1. Mechanical artifacts.
2. Changes in "driving forces".
3. Changes in permeability.
4. Changes in other unknown parameters.

1. Of possible mechanical artifacts variations in the extracardiac surface film (O_R) and deformation of the extracellular space due to "squeezing" can be excluded as causes of the results obtained for Na^{24} and Br^{82}. One possibility which, however, cannot be neglected is that the mechanical activity of the preparation affects the extracellular matrix so that its physico-chemical properties are altered, e.g. for diffusion.

2. The cycles of electrical activity may be coupled with changes in the electro-chemical and electro-osmotic conditions within the excitable tissue. The "action potential" for example could be accompanied by reduced electro-osmotic forces which would result in an outward "bulk" flow (cf. Teorell, 1960). According to the "electrokinetic excitability analogue system" described by Teorell (1960, 1962) there is a "total net salt flux" which from the instant that depolarization begins is out of the cell, and which just before or during repolarization changes to a gradually increasing influx. With the next depolarization it reverses again to an efflux (spontaneous repe-

[1] Danielson (1964) studied the SO_4^{--} distribution in toad heart. The SO_4^{--} spaces were for sinus venosus 63 ($\pm$6.5), atrium 49 ($\pm$4.0) and ventricle 22 ($\pm$2.5) ml per 100 g wet tissue respectively.

titive excitation). These results have recently been confirmed by ionic tracer flux analysis on the "membrane oscillator" described above (Teorell, 1964).

3. Permeability changes associated with the electrical activity cycles seem a possibility. According to Hodgkin and Huxley (1952 *a–d*) there should be a "sodium inward current" associated with depolarization caused by an increase in permeability for Na.—As can be seen, however, in Figs. 47 and 48 the radioactivity increase in the preparation (influx into the cells?) begins *before* depolarization. It should be pointed out that the time relations between the electromechanical activity and the radioactivity pattern recorded, shown in Figs. 26 (p. 56), 47 and 48, represent only one of the boundary limits for this relationship (cf. p. 81); any shift of the radioactivity pattern should be to the left in the figures. It is possible that the early inward sodium movement is connected with the spontaneous electrical activity of the sinus venosus, one of the features of which is the slow depolarization of the pace-maker and near pace-maker regions (cf. Figs. 4 and 5, p. 19).

The Br^{82} activity curve (Fig. 26, p. 56) for the "mean cycle" may also be regarded as a possible analogue for the Cl^- activity curve (cf. p. 50). As can be seen in Fig. 26 the Br^{82} activity is maximal during the electrical activity, rapidly decreasing during the steep slope of repolarization becoming minimal during most of the diastolic phase. From potential and resistance measurements (Purkinje fibres) Carmeliet (1961) claimed that there is an appreciable inward current of chloride ions occurring directly after the crest of the action potential and persisting during the plateau. The direction of the chloride ion movement during the slow diastolic depolarization was not clear, but, according to Carmeliet, there were two possibilities: 1. The chloride ions were moving inwards and exerted a stabilizing effect, and 2. the chloride ions were moving outwards and favouring depolarization. — As can be seen, however, in Fig. 26 the radioactivity increase in the preparation (influx into cells?) begins *before* depolarization (cf. time relation above); this being the case it may be connected with the spontaneous electrical activity of the sinus venosus. The minimum value of the Br^{82} activity during the main part of the diastolic phase should in this case favour depolarization.

An *explanation* of the radioactivity pattern for the mean cycle must, however, await further experimentation.

GENERAL SUMMARY

The aim of the present work was to study the relationship between ion fluxes and the single electro-mechanical cycle in the sinus venosus and atrium of toads.

Chapter I. The purpose of the present investigation

A short discussion about the current theories for the genesis of resting and action potentials in excitable tissue, together with a review of certain relevant experimental results in the literature, are presented. The greater part of the works cited concern information about the ion fluxes per activity cycle obtained from studies of many cycles. Only in isolated cases has the flux during a single cycle been studied. There is as yet no uniform picture of the relation between ion fluxes and electrical activity, based on solid experimental evidence. The main difficulty seems the lack of suitable experimental methods. The aim of the present work was to make a contribution to both the biological and methodological aspects of this problem. In order to study the direct relationship between the single electro-mechanical cycle and the ion flux, two main methods were used: an *influx method* and an *efflux method*.

Influx studies: The radioactive uptake by sinus venosus was measured discontinuously at short time intervals in a perfusion chamber placed in the well of a scintillation crystal. The preparation was alternately perfused with Ringer containing a gamma emitting isotope of the ion being studied (100 msec) and then with a hydrophobic silicone oil (1000 msec) which served to wash away the radioactive perfusing fluid, so that the activity of the preparation could be determined. The electrical changes were followed continuously with a surface electrode (ECG). Heavy radiactive shielding was required.

Efflux studies: The heart preparation was loaded with radioactivity and washed by a stream of inactive Ringer solution ("wash-out" technique). Two samples of the efflux were collected each second, and good resolution in respect to time was obtained.

Chapter II. The sinus venosus and the atrium of the heart of the toad

The macroscopic and microscopic anatomy and the electrical characteristics of the tissue are described. For both influx and efflux studies the preparation was turned inside out so that the endothelial surface lay outmost and was bathed directly by the perfusing fluid. In the ion flux studies made it was very important that the electrical and mechanical activities should be very nearly synchronous in all parts of the preparation. Preliminary studies showed that at the low temperature used for the experiments the electrical activity could be regarded as being virtually synchronous throughout the preparation, and that the ECG could be used as a reference point for determining the length of this activity. The mechanical processes had a constant relationship to the electrical cycles.

Chapter III. The perfusion system

The perfusion system and the critical conditions required for the influx and efflux experiments are described. It was found that for the influx experiments a mean linear velocity of about 300 cm/sec (perfusion volume 7 ml/sec) corresponded to about 400 changes/sec of the perfusion fluid around the preparation which had a wet weight of about 20 mg; at this flow velocity both Ringer and silicone oil flows were strongly turbulent. Turbulent flow was required both for good exchange properties between the Ringer and the preparation and for efficient removal of the Ringer during the silicone oil period.

Chapter IV. Methods

The recording methods (ECG, action potential, mechanogram and radioactivity) are described. In the influx method (direct monitoring method, DMM), great demands are made on the recording system, since no individual measurement can be repeated. Two separate analysis systems were therefore used: 1) a continuous "decimal-pip" system and 2) a discontinuously working digital printing system. The accuracy of the radioactive analyses was adequate.

Chapter V. Procedure

Two types of influx methods were used: 1) alternate perfusion of the preparation with Ringer and silicone oil and analysis of the radio-

activity during the silicone period (direct monitoring method, DMM); 2) determination of the amount of isotope or other test substance taken up from a soaking bath. In the efflux experiments (sampling method, SM), two samples of a perfusate which had flowed past a preparation loaded with the test substance or isotope were collected each second.

Chapter VI. Some influx experiments; the calculations and the graphical representation of such experiments

Two influx experiments (DMM), one for Na^{24}, the other for Br^{82}, on isolated sinus venosus are described. In both experiments the curves relating activity to time consist of at least two components: 1) a mean exponential influx curve (DC component) on which are superimposed, 2) oscillations (AC component). During one of the Na^{24} influx experiments the sinus venosus stopped beating, and the DC component then exhibited another time course (the rate of increase of radioactivity of the preparation decreased). The radioactivity was measured every 1.2–1.3 sec; this is in relation to the biological process rather long, and from this point of view the influx method can be regarded as rather coarse. For this reason the radioactivity pattern differed greatly from cycle to cycle, which was not unexpected because in addition to any systematic fluctuations the AC component (single measurements) also reflects all the random variations, biological and non-biological, i.e. instabilities in the perfusion system and recording systems, and statistical counting errors of the radioactivity. In order to determine the true systematic pattern, a type of continuous averaging known as the "computing of average transients", *c-a-t*, was used. The assumptions necessary in order to calculate a *c-a-t* are described, together with the details of the procedure. By means of this procedure it is possible to calculate a mean heart cycle, and though certain reservations must be made, this seems a useful procedure for extracting information.

Chapter VII. Some methodological problems of the influx experiments

A. The background activity and "trapped activity"

A sham influx experiment without a preparation in the perfusion chamber was made, and the radioactivity pattern determined. There are two sources of background activity: 1) That arising extraneously from radioactivity in other parts of the perfusion system which could

not be entirely screened off, and 2) that arising from some radioactivity that during silicone oil perfusion in the chamber is not been washed away. This latter ("trapped activity") was estimated to correspond to about 0.7 ml of active Ringer solution.

B. The surface layer of the heart preparation during Ringer and silicone oil perfusion

It is concluded that during Ringer perfusion the tissue is surrounded by an unstirred layer, probably about 0.5–7 μ thick (this unstirred layer does not seem to be a rate-limiting step in the movements of tracer between the Ringer solution and the tissues). During silicone oil perfusion following the passage of the Ringer-silicone phase boundary, an extra-cardiac surface film of active Ringer solution (O_R) is left behind. At the beginning of the silicone oil perfusion period this has the same specific activity as the Ringer solution (the volume of O_R was calculated to be equivalent to about 8.5 % of the wet weight of the preparation, corresponding to a maximal thickness of 25–30 μ). The total radioactivity in the perfusion chamber during a single silicone oil period of about 1 second was practically constant.

C. The uptake of tracer in the tissue of the preparation during Ringer and silicone oil perfusion

Evidence is presented that the exponential increase of radioactivity with time in the preparation is representative for the mean activity in the tissue. During the alternate Ringer-silicone oil periods the tracer kinetics are different; during Ringer perfusion the source of radioactivity is infinitely large, while during the silicone oil perfusion period it is rather limited. The course of the exponential curve depends on the electro-mechanical activity of the tissue and the perfusion programme (ratio between the lengths of the Ringer and silicone oil perfusion periods). The AC component (mean cycle) exhibits systematic fluctuations in relation to the electro-mechanical cycle of the spontaneously beating preparation. These systematic fluctuations may possibly arise from non-biological (biological = transmembrane ionic movements) in two ways: 1) the mechanical activity may distort the external surface film (O_R) during silicone perfusion and 2) electro-mechanical activity may produce changes in the extracellular space. Further, it is pointed out that a shift of the mean cycle with respect to time cannot be great (not more than 1.3 sec).

D. The equilibration volumes and uptake rates during an influx experiment

The equilibration volumes were determined by soaking the tissue in a solution containing Na^{24}, Br^{82} and raffinose; the latter was used as an indicator for the extracellular space. The uptake of raffinose in the sinus venosus and atrium was slower than that of Na^{24} and Br^{82}. From these values it was concluded that during the influx experiments made with the DMM at a temperature of $+5°C$, Na^{24} and Br^{82} moved primarily into the extracellular space (the equilibration time for the sinus venosus was about 200–250 sec during continuous Ringer perfusion). The influx of the isotopes in the DMM was faster than in a simple soaking technique.

E. Studies of the distribution of K^+ ions between the perfusion solution and the outermost cells of the tissue preparation

The time between the contact of the sinus venosus preparation with a Ringer solution containing 44 mM K^+ and the depolarization could enable an estimate to be made of the proximity of the outermost cells to the perfusion solution. The time lag measured in this way thus was interpreted as the time required for the K^+ ions to reach the cellular membranes in sufficient concentrations to produce depolarization.

F. Electro-mechanical dissociation, the effect of the mechanical activity on the influx (DMM) patterns for Na^{24} and Br^{82}

Mechanical activity may possibly affect both influx and efflux studies by causing variations in the surface volume relationship of the tissue during the contraction phase. These in their turn may generate mechanical artifacts by 1) altering the geometry of the extracellular space and 2) distorting a critical surface film surrounding the preparation. Lowered mechanical activity without essentially altered electrical activity (electro-mechanical dissociation) can be achieved by low Ca^{++} concentrations. When the Ca^{++} concentration was lowered to 0.5 mM the contraction was then only 25 % of the normal amplitude (isometric). As yet no influx experiment has been made on such a preparation, but the efflux has been studied.

Chapter VIII. The efflux of tracer from the sinus venosus and atrium under different perfusion conditions

To ascertain the importance of the perfusion conditions, the relationship between perfusion velocity and the efflux pattern was studied in both spontaneously beating and inactive preparations. It was found that the efflux depends on the perfusion velocity and is different for different ions. Only when the perfusion velocity was low were cyclical variations in the amount and concentration of tracer in the perfusate correlated to the electro-mechanical activity of the spontaneously beating preparation (depolarization caused increased efflux of tracer). At a high perfusion velocity (linear mean flow $\bar{v} \approx 300$ cm/sec) corresponding to the perfusion conditions of the influx experiments (DMM), there were no fluctuations in concentration or amount in the perfusate related to the electro-mechanical activity.

If "extracellular squeezing" were an important factor in the efflux, this should also be seen with high perfusion velocities. The results obtained rather suggest that the distortion of the unstirred surface layer surrounding the preparation is important; such an "external squeezing" effect would be expected to cause variations in the efflux correlated with the mechanical activity. One of the difficulties in obtaining clear-cut results with an efflux method, even under optimal perfusion conditions, are that the duration of the experiment is severely limited as the radioactivity soon becomes reduced to unpracticable levels. Because of this a continuous averaging technique as used for the influx experiments cannot be used to eliminate "background noise".

General discussion

With perfusion at a high linear mean velocity ($\bar{v} \approx 300$ cm/sec) there were no definite fluctuations in the efflux of Na^{24} or Br^{82}, from a spontaneously beating heart preparation, which were correlated with the electro-mechanical cycles. In influx experiments (direct monitoring method), however, both isotopes exhibited fluctuations in relation to the single heart cycle. The interpretation of this observation is discussed. The tissue is both structurally and functionally particularly complex; the electrical (action potential) and the mechanical (contraction) activities run in parallel and are difficult to dissociate from each other. Tracer studies on such a preparation can-

not *a priori* be expected to give comprehensible results which reflect the details of ion distribution in the tissues (between the intra- and extracellular spaces). The possible effects of mechanical activity are discussed further, and it is suggested that an "external squeezing" effect by the distortion of a critical unstirred surface layer surrounding the preparation may be responsible for some results reported in the literature of an increased tracer efflux associated with the electro-mechanical activity.

The DMM, with which a large number of cycles are analysed, makes it possible by a continuous averaging method to calculate a mean cycle. This increases the sensitivity of the method enormously. *It is even possible to study tracer fluxes so small that they lie within the uncertainty of the analysis method. The resolution with respect to time for DMM is relatively good* (this depends on how short the Ringer and silicone oil perfusion periods can be made, and is limited for technical reasons). It should be pointed out that DMM always gives tracer flux into the preparation as a whole, but as long as internal concentration gradients for the specific activities exist, calculations of total flux cannot be made.

The mean cycle was calculated for both Br^{82} and Na^{24} fluxes. There was a maximum for the recorded activity at or immediately after depolarization, while the minimum occurred in the diastolic phase. The systematic fluctuations may depend either on a correlation between the size of the extra-cardiac surface film (O_R) during silicone oil perfusion and the mechanical activity cycles or an electro-mechanical effect on the properties of the extracellular space. It is concluded that variations in O_R associated with the electro-mechanical activity can be excluded as a cause of the oscillations in the mean cycle. It is also not clear how "extracellular squeezing" could bring about such a correlation. *If a purely mechanical basis can be excluded, the question arises as to whether the results for Na^{24} and Br^{82} are biological realities. If this is the case it would mean that a Na influx began before depolarization. This question and the result for Br^{82} for the mean cycle are briefly discussed.*

Acknowledgements

My thanks are due to the members of the scientific staff of the Institute of Physiology, University of Uppsala, for many valuable discussions and helpful criticism.

I would also like to thank Mrs. M. Waller who made all the drawings, Miss U. Sjöberg for technical assistance and Mr. B. Fors, Mr. S. Norberg, Mr. A. Persson, and Mr. L. O. Filmberg for help with the construction of the equipment. Mr. O. Wistrand and Mr. A. Karlsson have also given me much help for which I express my sincere thanks.

Financial support was provided by the Medical Faculty of the University of Uppsala, the Swedish Society for Medical Research, Magnus Bergwall's Foundation, Swedish National Association against Heart and Chest Diseases, "Svenska Personal-Pensionskassan", "Anna Maria Lundins Stipendiefond", a PHS research grant H-5346 from the National Heart Institute, Public Health Service, U.S.A., and a research grant B-3712 from the National Institute of Health, U.S.A.

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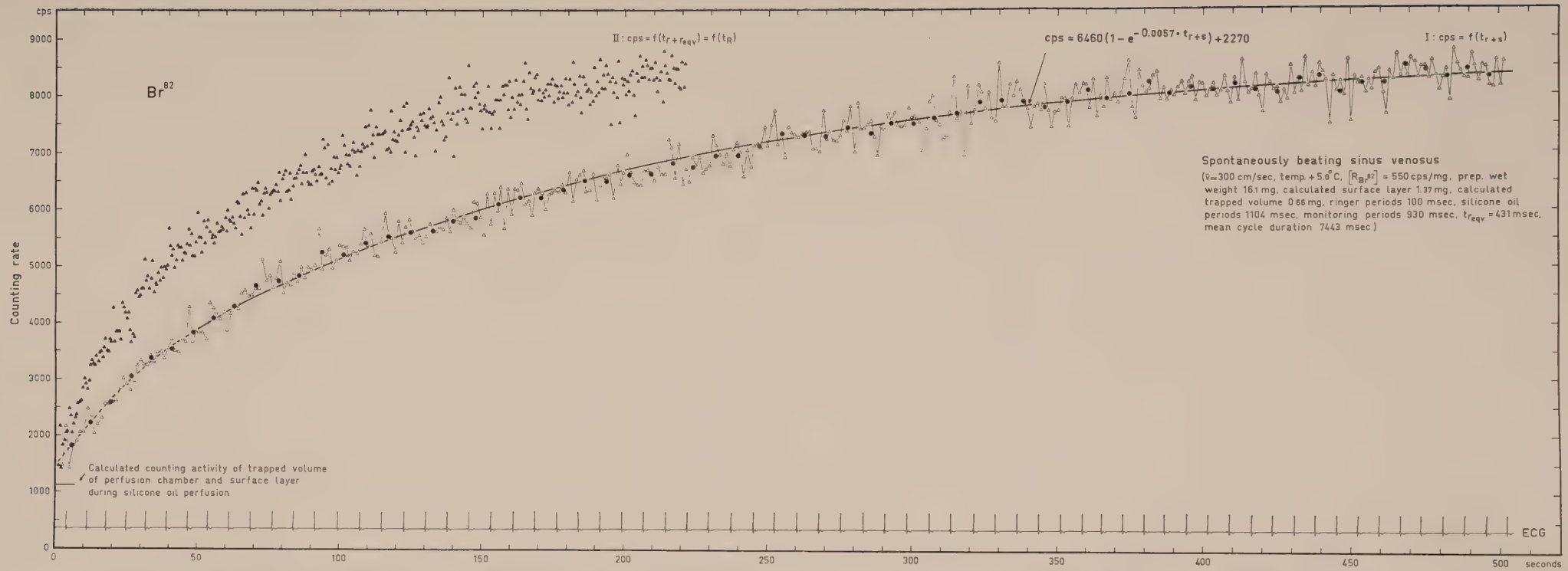

FIG. 25. An influx experiment for Br^{82} on an inverted, spontaneously beating sinus venosus (DMM). The radioactivity recorded during each monitoring period has been assigned to the time point at the end of the preceding Ringer period (open triangles). The solid circles are the mean values for the activities recorded during each heart cycle. The mean trend line for the increase of radioactivity with time was calculated as described in the text (curve I). Curve II is the estimated course of the radioactive increase if perfusion had been made continuously with active Ringer solution (time scale $t_r + t_{r_{eqv}}$ instead of $t_r + t_o$; see p. 88). The ECG is also shown.

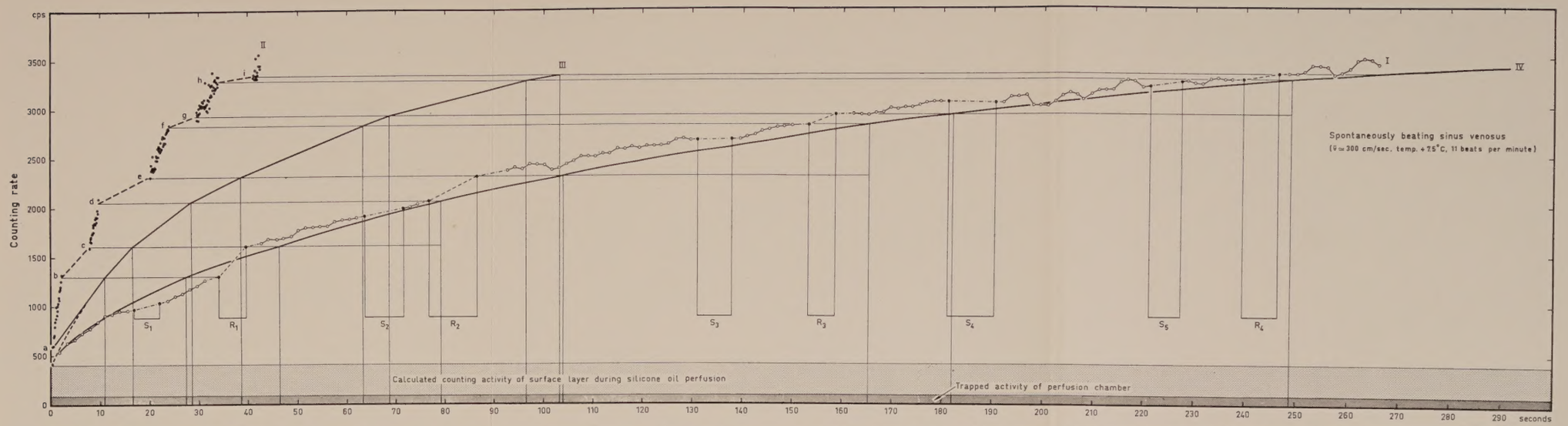

FIG. 32. Influx experiment for Na^{24} on an inverted, spontaneously beating sinus venous. Perfusion and analysis programme (DMM): $t_r = 100.4$ msec, $t_s = 1320$ msec, $t_m = 1001$ msec; the alternate Ringer-silicone periods were interrupted by either long Ringer or silicone periods (R_1, R_2, R_3, R_4 and S_1, S_2, S_3, S_4, S_5). Active Ringer solution ESA 1.43 kcps Na^{24}/μmole Na. The wet weight of the preparation was 23.4 mg. "Trapped activity" in the perfusion chamber corresponded to 0.42 mg of active Ringer solution and was determined by perfusion with the chamber empty (see Fig. 27, p. 62). For further details see text.

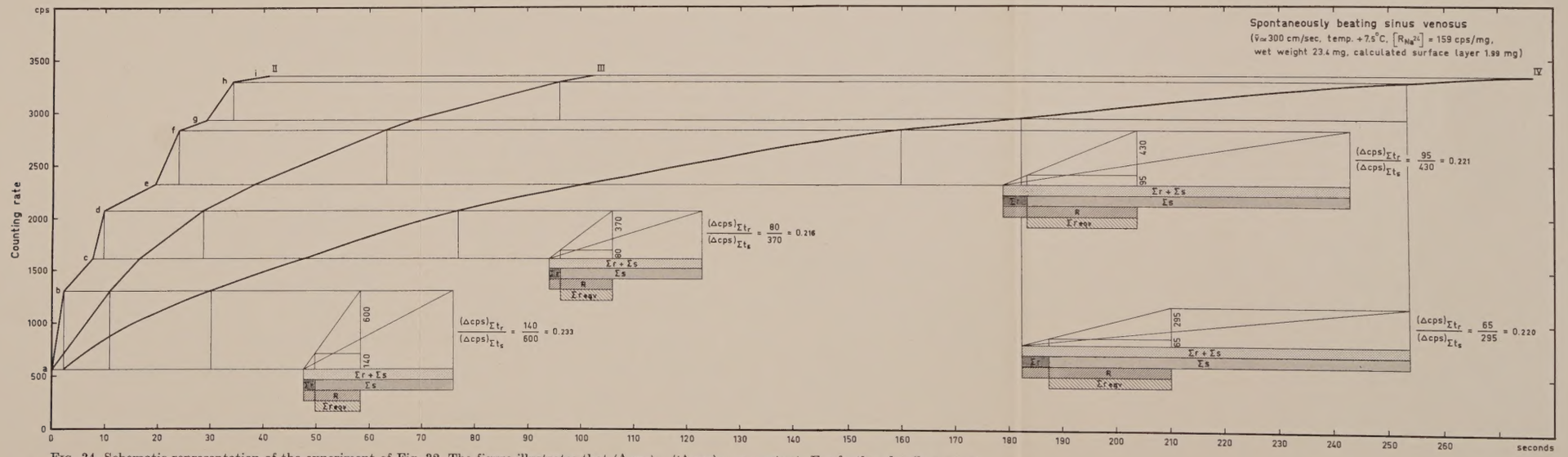

FIG. 34. Schematic representation of the experiment of Fig. 32. The figure illustrates that $(\Delta \text{cps})_{\Sigma t_r}/(\Delta \text{cps})_{\Sigma t_s} \approx$ constant. For further details see text.

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